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COLORIMETRIC METHODS OF ANALYSIS

Including Photometric Methods

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PREFACE

Volume IIIA brings the organic photometric methods of analysis up to date without repetition of the details in Volume III. It supplies the new material which has appeared since about August 1953, subject to the delays in publication and distribution of the original journals.

The advances in photometric determination of organic compounds have been somewhat greater than those in the inorganic field. Seldom does a new insecticide, fungicide, or drug appear without there being ortly thereafter a photometric method for its determination. This is e more true because the modern laboratory commonly contains an inrument for reading in the ultraviolet as well as the visible range. Therefore, ultraviolet methods of reading, often without addition of a reagent, have become more prevalent.

The authors of Volume IIIA are the same as for Volume IIA. The senior author, Dr. Foster Dee Snell, has prepared and edited the final manuscript. Dr. Cornelia T. Snell has collaborated as in the previous edition. Dr. Chester A. Snell has contributed from his experience as Director of the Analytical Department of Foster D. Snell, Inc. Mrs. Carol P. Cohen has contributed valuable editorial assistance and Miss Helen Nettleton splendid secretarial cooperation.

New York, N. Y.
May, 1961

Foster Dee Snell
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CHAPTER 1

INTRODUCTORY

In Volume III this chapter consisted of miscellaneous comments about the organization of the book together with instructions for its use. The comments and instructions merit repetition here.

The volume of literature and the number of individual substances to be covered are so great that every possible method of condensation has been applied. Thus a characteristic ending of a procedure will be: "Read at 560μ against a reagent blank." It is not felt that one need add thereafter: "Compare with a calibration curve prepared under the same conditions." Similarly, unless there is something unique about the preparation of a blank, it is not described. Ordinarily the color developed is suitable for colorimetric estimation. This fact will not be mentioned unless there is some reason to do so: for example, when the color that appears first cannot be read, but changes to one that can. Similarly, if Beer's law holds, this will not be mentioned unless there is some reason for it, as when the law holds over a limited concentration range only. It is our belief, or at least hope, that this will not reduce the usefulness of the book, although it will reduce an otherwise necessarily large bulk.

It has not seemed worth while to specify the use of a "250-ml. beaker" or a "250-ml. Erlenmeyer flask" for carrying out a reaction. Often some other container may be more available and will be used. Thus, if a 10-gram sample is digested with 150 ml. of water at 70°, it is assumed (1) that it will be put in a container and (2) that the container will be put in a water bath or an oven. Of course, if special conditions to protect from oxidation are necessary, they are included.

Few volumetric flasks are mentioned. If a 100-mg. sample is dissolved in acetone and diluted to 10 ml., the operator will naturally dilute with an accuracy comparable to that desired. For control work of a "go-no-go" type, dilution may be in a graduated cylinder. Usually dilution will be in a volumetric flask. By this form of expression the typographers have missed many hundreds of mentions of such flasks, and many thousands of words as a result.

As an example the phrase "Transfer to a 100-ml. volumetric flask and dilute to volume with distilled water" is replaced by "Dilute to 100 ml. with water."

Methods of specification of solutions vary widely. For example, when a N solution of sodium hydroxide is mentioned there is usually no criticality as to whether it be 0.9 or 1.1 N: often it is added to obtain a rough end point. Hence, usually such solutions have been given in per cent, a 4% solution rather than a N solution. The same applies to acids often prepared by dilution, 1:35 sulfuric acid rather than normal. If no concentration is mentioned, the common commercial concentration is meant; for example, ethanol means 95% ethanol, and concentrated sulfuric acid means that of density 1.86.

No calibration curves are reproduced because they must be prepared with the reagents being used. Aside from the space they occupy, if present there is a temptation to assume that maybe they will be accurate enough. Standard solutions for calibration are rarely shown, it being left to the worker's option to select such concentrations as will be applicable to his specific problem.

Much of the same basic principle applies to weights cited. A reference may call for 1, 1.0, or 1.00 mg. The accuracy of taking the sample depends on the purpose of the analysis rather than on an arbitrary indication of accuracy possibly desirable. Hence, with rare exceptions, volumes or weights cited do not go beyond the last numeral or the decimal point, whichever is the smaller. The worker can readily select the accuracy needed for his purpose. Whether it is attainable is another subject.

Problems of classification are complex. Thus there is a chapter on sterols. If classified entirely by structure, the majority of hormones would appear there. Yet the hormones are so closely integrated as a unit that to break them up would be unwise. Should vitamins appear together? There is no direct relationship between thiamine and ascorbic acid. Should thiamine appear with aliphatic amines and ascorbic acid with substituted aliphatic monobasic acids? Should acetoacetic acid appear as a substituted monobasic acid or as a ketone? The net conclusion is to classify primarily by chemical structure when there is not a good reason to do otherwise as in the case of the hormones. But there are so many exceptions that the simplest way to find a compound is through the index rather than thumbing through the chapter where it might be.

Details of infrared and ultraviolet determinations are rarely given, an exception being vitamin A where the spectrophotometric method has become USP. The reason is that a line has to be drawn somewhere to encompass the material in a single volume. That was selected as the fringe with references often given to such methods.

With the heterogeneity of expression of weights in grams, milligrams, micrograms, etc., an effort has been made to translate all to grams and milligrams, even though fractional in terms of the latter. For good reasons there are a few exceptions, usually because the amounts are fractions of even gamma.

CHAPTER 2

HYDROCARBONS

THE DEVELOPMENTS since publication of the third edition are surprisingly great. They include acetylene in ethylene oxide as copper acetylide, substituted ethylenes as iodine complexes, propylene as acetone, nitrated benzene with various reagents, and direct reading of styrene and polystyrene. Biphenyl is isolated from citrus oil by chromatography for direct reading. Benzo[*a*]pyrene is read directly. Isolation of carotenoids by chromatographic methods has made great strides. Menthane has an absorption peak at 292 mμ.[1]

When separated by elution from aluminum oxide of limited activity the prominent peak absorption for various hydrocarbons is at the wave lengths shown in Table 1.[2]

The usual methods for determination of toluene are by nitration and development with a ketone (Vol. III, pp. 15-7, see Vol. IIIA, methods preceding). As a variant the nitration product is extracted with benzene and gives a violet color in the presence of alkali.[3] This method is accurate to 3% and benzene and chlorobenzene do not interfere.

When light from a hydrogen-discharge tube is passed through xylene vapor, characteristic absorptions are obtained for *o*-xylene, *m*-xylene, and *p*-xylene in the ultraviolet.[4] The wave lengths are 268.6 mμ, 270.5 mμ, and 272.2 mμ, respectively. The results show an average precision of ±10%. The minimum amount of isomers so determined are: 5% for ortho, 1% for meta, and 0.1% for para.

Anthracene (Vol. III, p. 27) in commercial carbazole is read at 358 mμ with ethanol as the solvent.[5] If the sample solution stands in ethanol longer than a day the absorption intensity decreases. Alternatively the carbazole is read 290 mμ and the anthracene at 332 mμ.[6]

---

[1] Adolfo Leandro Montes, *Anales asoc. quím. argentina* 42, 30-7 (1954).

[2] A. J. Lindsey, *Anal. Chim. Acta* 20, 185-181 (1959); 21, 101-4 (1959).

[3] W. Grabowicz, *Med. Pracy* 6, 297-302 (1955).

[4] Alain Berton, *Ann. chim.* 19, 394-404 (1944); *Compt. rend.* 236, 1679-81 (1953); Cf. A. Miss and C. Lupu, *Compt. rend. congr. intern. chim. 31E, Liège, 1958* 1, 593-600 (1959).

[5] T. Yoshida, O. Hibino, H. Suzumura, and N. Tamura, *Coal Tar (Japan)* 7, 114-6 (1955).

[6] J. Świetoslawska and H. Waledziak, *Chem. Anal.* (*Warsaw*) 1, 246-51 (1956).

TABLE 1. WAVE LENGTHS OF MAXIMUM ABSORPTION FOR VARIOUS HYDROCARBONS

Wavelength	*Compound*
471	Naphthacene
441	Naphthacene
437	Perylene
430	Anthanthrene
409	Perylene
404	3 : 4-Benzpyrene
401	1 : 2, 3 : 4-Dibenzpyrene
394	3 : 4, 9 : 10-Dibenzpyrene
385	3 : 4-Benzpyrene
384	1 : 12-Benzperylene
380	Anthanthrene
376	Anthracene
372	3 : 4, 9 : 10-Dibenzpyrene
364	3 : 4-Benzpyrene
363	1 : 12-Benzperylene
357	Anthracene
353	Azulene
350	1 : 2, 3 : 4-Dibenzanthracene
344	3-Methylpyrene
341	Azulene
340	Acenaphthylene, Anthracene
339	Coronene
335	Acenaphthylene, Pyrene
333	1 : 2, 3 : 4-Dibenzanthracene
332	1 : 2-Benzpyrene
331	1 : 2, 3 : 4-Dibenzpyrene
323	Acenaphthylene, 1 : 2, 3 : 4-Dibenzanthracene
321	Acenaphthene
320	2-Methylnaphthalene, Pyrene, Chrysene
317	1 : 2-Benzpyrene
311	Naphthalene
307	Acenaphthene, Anthanthrene
306	Chrysene
305	2-Methylnaphthalene
302	Coronene
301	Fluorene
300	Acenaphthene, 1 : 12-Benzperylene
297	3 : 4-Benzpyrene
295	3 : 4, 9 : 10-Dibenzpyrene
294	Chrysene
293	Phenanthrene
290	1 : 2-Benzpyrene, 1 : 2, 3 : 4-Dibenzpyrene
289	1 : 2-Benzanthracene, 1 : 12-Benzperylene
288	Acenaphthene, Fluoranthene
285	3 : 4-Benzpyrene
278	1 : 2-Benzanthracene, 1 : 2-Benzpyrene
276	3-Methylpyrene
273	Pyrene
270	Naphthacene
269	Chrysene
266	3 : 4-Benzpyrene
265	Fluorene
261	Benzene
255	Benzene
253	Anthracene, Perylene
252	Phenanthrene
249	Benzene
243	3-Methylpyrene
242	3 : 4, 9 : 10-Dibenzpyrene
237	Fluoranthene
233	Anthanthrene
221	Fluorene

ACETYLENE

The red cuprous acetylide, produced by the reaction between acetylene and an ammoniacal solution of cuprous sulfate (Vol. III, p. 5), is stabilized by isopropanol with gelatin as a protective colloid.[7] Concentrations of acetylene in ethylene oxide down to 0.001% are determinable with a reproducibility of the order of ±5% of the determined acetylene content. Alternatively, copper nitrate is used as reagent, the copper acetylide filtered, washed, dissolved in ammonium hydroxide, and read as the blue copper complex.[8] Also, silver acetylide is precipitated and read turbidimetrically.[9]

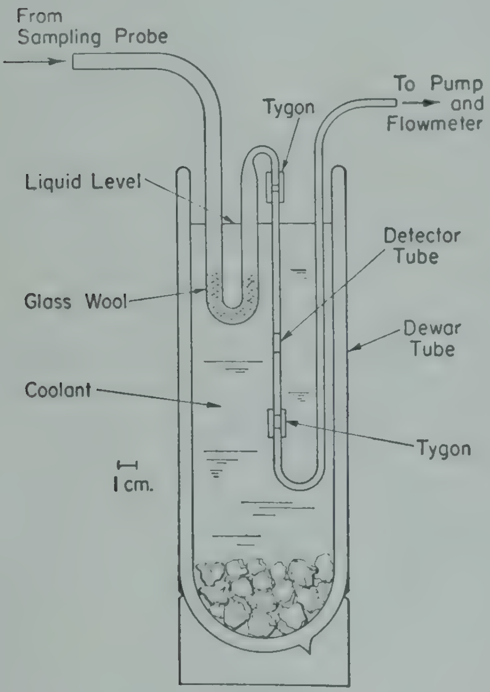

FIG. 1

Apparatus for absorption of acetylene

In another version of the copper acetylide method it is developed directly on the silica gel.[10] This is applicable in the range of 0.1-10 ppm. by adsorption of the acetylene from a gas stream on the silica gel cooled to −78°, warming to room temperature, and treatment with an ammoniacal cuprous chloride to form the precipitated copper acetylide. The method is applicable for 0.001 ppm.

Standards may be made with acetylene mixed in air followed by sealing the tube after color development. The color of the cuprous acetylide on silica gel is also duplicated by a combination of azolitmin, litmus, methyl red, and oil red, or by the use of color chips of the type used with carbon-monoxide indicating tubes. The sample tubes are allowed to stand at room temperature for 3 minutes after cooling to allow the acetylene to diffuse evenly through the gel. For concentrations over

---

[7] V. W. Reid and D. G. Salmon, *Analyst* 80, 602-4 (1955).

[8] G. Almásy and I. Pallai, *Magyar Kém. Folyóirat* 59, 200-3 (1953).

[9] O. Kammori and Y. Babo, *J. Chem. Soc. Japan, Ind. Chem. Sect.* 58, 538-40 (1955).

[10] E. E. Hughes and R. Gordon, Jr., *Anal. Chem.* 31, 94-8 (1959).

1 ppm. a flow rate of 50 ml. per minute of sample is used. To extend the range to 0.01 ppm. of acetylene a flow rate of 200 ml. per minute is used. Concentration of acetylene in increments of 0.000012 mg. can be distinguished and the tubes may be stored before reading with only a slight loss of sensitivity after 4 hours.

Since the test is not specific but is given for any alkyne in which a triple bond occurs at the end of a chain, alkynes from 1-propyne to pentyne would interfere if present. Interference such as water and higher molecular weight alkynes are removed in a U-trap as shown in Figure 1. Hydrogen sulfide and some mercaptans interfere.

Procedure—*By ammoniacal cuprous chloride.* As reagent dissolve 1.5 grams of cupric chloride and 3 grams of ammonium chloride in 20 ml. of concentrated ammonium hydroxide and dilute to 50 ml. with water. For use mix 1 part of this solution with 4 parts of 10% hydroxylamine hydrochloride solution.

Prepare a tube with an outside diameter of 4 mm. and an inside diameter of 2 mm. Add a 2-cm. layer of 60-70 mesh glass beads. Follow this with silica gel which is free from iron oxides. This purified silica gel is obtainable from Mines Safety Appliance Company, Pittsburgh, Pennsylvania. After inserting it with a short length of thin-walled aluminum tubing through a funnel to a length of 1 cm., add another 1 cm. layer of the 60-70 mesh glass beads. Assemble the apparatus as shown in Figure 1.

Draw the gas to be analyzed through the tube, catching water and interferences in the U-tube. Use a flow rate of 50 ml. per minute for concentrations of acetylene over 1 ppm. and a flow rate of 200 ml. per minute for concentrations from 0.01 ppm. to 1 ppm. Let the tube stand 3 minutes after cooling to allow even diffusion of the acetylene. Treat with the reagent and compare with standards.

Ethylene oxide. As a reagent mix 10 ml. of 20% copper sulfate pentahydrate solution, 60 ml. of filtered 30% hydroxylamine hydrochloride solution, 30 ml. of 1:1 ammonium hydroxide solution, 50 ml. of 1% gelatin solution, and 50 ml. of water. Prepare fresh each day.

Pipet 10 ml. of reagent and 10 ml. of 99% isopropanol into a 50-ml. volumetric flask. Cool in an ice bath and add a suitable aliquot of sample using a cooled dipper pipet. Swirl in an ice bath. Bring to volume with distilled water and shake. Read at 550 mμ exactly five minutes after addition of the sample. Since ethylene oxide does not interfere, calibration curves can be prepared from pure acetylene.

Liquid oxygen.[11] Vaporize and absorb the acetylene in 5 grams of 17- to 20-mesh silica gel. Displace by a nitrogen flow at 400-100 ml. per minute into a mixed solution of 20 parts of 10% copper sulfate solution, 30 parts of 1:1 ammonium hydroxide, 60 parts of hydroxylamine hydrochloride, 50 parts of 1% gelatin solution, and 38.5 parts of water. Read at 517 mμ.

As silver acetylide. Absorb the acetylene by bubbling through 100 ml. of 4% silver nitrate solution in 1:30 ammonium hydroxide. Heat at 100° for 5 minutes to precipitate the silver acetylide and read the turbidity.

SUBSTITUTED ETHYLENES

Iodine and hydrocarbons undergo a reversible reaction to form addition complexes of the acid-base type.[12] Thus the iodine becomes an electron-acceptor and the hydrocarbon an electron-donor to reach an equilibrium defined by the law of mass action. This is applied to determination of mono-, di-, tri-, and tetra-substituted ethylenes in isooctane in the 275-350 mμ region of the ultraviolet spectrum.[13] The band for each type of substitution occurs at a characteristic wave length. Cyclopentene and cyclohexene react similarly to open chain olefines.

The maximum absorption characteristic of each olefin type is as follows:

$RCH{=}CH_2$	275 mμ
$R_2C{=}CH_2$	290-295 mμ
$RCH{=}CHR$	295-300 mμ
$R_2C{=}CHR$	317 mμ
$R_2C{=}CR_2$	337 mμ

Saturated hydrocarbons do not interfere. Aromatic hydrocarbons also form complexes with the iodine and since their absorption overlaps they interfere. Diolefines add iodine rapidly at room temperature[14] and interfere. Light, such as north light or that from fluorescent bulbs, catalyzes the formation of diiodo compounds which interfere. The rate of this reaction lessens as one goes toward the more fully substituted hydrocarbons. A small amount of such reaction, as distinguished from complex formation, destroys the desired relationship, vitiates the determination.

---

[11] R. Matsui, Y. Ito, E. Yasui, and H. Suzuki, *Kôgyô Kagaku Zasshi* **60**, 413-7 (1957); E. Yasui and H. Suzuki, *Ibid.* **61**, 173-9 (1958).

[12] H. A. Benesi and J. H. Hildebrand, *J. Am. Chem. Soc.* **71**, 2703-7 (1949); S. H. Hastings, J. L. Franklin, J. C. Schiller, and F. A. Matsen, *Ibid.* **75**, 2900-5 (1953).

[13] L. J. Andrews and R. M. Keefer, *Ibid.* **74**, 458-60 (1952); D. R. Long and R. W. Neuzil, *Anal. Chem.* **27**, 110-4 (1955).

[14] S. Freed and K. M. Sancier, *J. Am. Chem. Soc.* **74**, 1273-5 (1952).

In the concentration used only about 2% of the iodine present is complexed. In the region used the background absorbance of the excess iodine is less than 0.2.

Procedure—To purify the isooctane, shake thoroughly with concentrated sulfuric acid and percolate one liter through a 3-foot column, 1 inch in diameter, containing 200-mesh silica gel. This removes traces of aromatics and dienes. As so purified the absorbance must be less than 0.05 down to 230 mμ in a 1-cm. silica cell. Dissolve the olefin in the purified isooctane so that the concentration is approximately 0.1 mole per liter. Add 5 ml. of the olefin solution to 5 ml. of 0.1% iodine solution in isooctane. Read within 1 minute at 400-250 mμ in a 1-cm, silica cell using a temperature standardized within ±1°. This minimizes the catalytic effect of light. Alternatively the mixed sample and reagent may be stored in the dark for at least an hour without change.

If necessary to keep the absorbance of the complex between 0.3 and 1.0, dilute the olefin solution before adding the iodine, and repeat the determination. Dilution of the final iodine-olefin complex is not satisfactory since the iodine concentration must be constant. To correct for uncomplexed iodine, read 5 ml. of 0.1% iodine solution in 5 ml. of isooctane. The isooctane gives no blank.

Calculate weight absorbancy as follows:

$$a' = A_c/(C_1) \times (C_0)$$

a' = weight absorptivity

A_c = absorbance at λ maximum, corrected for iodine background

(C_1) = concentration of iodine in the final solution in grams per liter

(C_0) = concentration of the olefin in the final solution in grams per liter.

With two or more members of the class of compounds present, correct results by the usual linear simultaneous equations.

PROPYLENE

Propylene is determined by conversion to isopropanol, oxidation to acetone, and condensation with furfural to give a red-violet acetone-difurfurylidene condensation product.[15] The amount present in ethylene can also be determined by the yellow color produced when passed through concentrated sulfuric acid.[16]

---

[15] Henri Etienne, *Ind. chim. belge* **17**, 455-8 (1952).

Procedure—To convert propylene to isopropanol, evacuate a 12-liter flask to about 500 mm. of mercury and charge with 100 ml. of 80% sulfuric acid containing 1 gram of mercuric oxide. Add 10 liters of gas and agitate vigorously for 30 minutes. Cool the contents by adding about 800 grams of ice. Distil off 100 ml. The propylene is present in this distillate as isopropanol.

Reflux a 25-ml. aliquot of the distillate, which should contain 0.025-0.5 mg. of isopropanol, with 25 ml. of a mixture containing 60 parts of potassium dichromate, 80 parts of concentrated sulfuric acid, and 270 parts of water. After 30 minutes the isopropanol will have been quantitatively oxidized to acetone. Distil off 50 ml. and use a 5-ml. aliquot for determination of the acetone by reaction with furfural, page 278.

DIVINYLACETYLENE

Acetone and divinylacetylene are read separately or together in the ultraviolet in vinyl acetate. Each must be corrected for the other.[17] Appropriate concentrations are 0.05-0.5% of acetone and 0.00005-0.003% of vinylacetylene.

Procedure—*Acetone and divinylacetylene in the ultraviolet.* The sample is dissolved in vinyl acetate. Read at 275 mμ and 266 mμ.

$$\% \text{ acetone} = 0.0002627(1870D_{275} - 393D_{266}).$$
$$\% \text{ vinylacetylene} = 0.0002627(2.50D_{266} - 2.33D_{275}).$$

BENZENE

After nitration, the color of *m*-dinitrobenzene and 2,4,6-trinitrotoluene is developed by potassium hydroxide in acetone-benzene with pyridine and sodium hydroxide, or by butanone (Vol. III, p. 14). Toluene is determined at the same time. If the nitration is conducted in 50% acetic acid with sodium nitrite and hydrogen peroxide, the color in alkaline solution does not require addition of a ketone for development. Benzene in ethanol is read directly in the ultraviolet.

When benzene-monochlorobenzene are nitrated the *m*-dinitro derivative is obtained in each case. That of benzene has a maximum absorption at 570 mμ but that of chlorobenzene is weaker and at 540 mμ. They are differentiated by boiling with sodium carbonate solution to convert the chloro compound to 2,4-dinitrophenol absorbing at 420 mμ.[18]

---

[16] Z. I. Matveeva and G. A. Sokolova, USSR patent 105,545 (1957).

[17] C. Capitani, E. Milani, and E. Peccatori, *Chim. e ind.* (Milan) 38, 102-S (1956).

Procedure—*By butanone. Gases for benzene and toluene.*[19] The conditions given here so minimize the compounds other than *m*-dinitrobenzene and 2,4,6-trinitrotoluene that in the range used they may be disregarded. The 2,4,6-trinitrotoluene formed has only one-third the maximum of *m*-dinitrobenzene at 565 mμ used for reading the latter. Likewise *m*-dinitrobenzene has only one-tenth the absorption of 2,4,6-trinitrotoluene at 462 mμ used for the latter. The maximum for 2,4,6-trinitrotoluene is reached in 15-20 minutes and then fades rapidly. The maximum for *m*-dinitrobenzene is reached in 20 minutes and stable for 45 minutes. Conditions of shaking affect the color of sample and standard and must be the same for sample and standard.

Absorb the benzene and toluene as their nitro derivatives in 10 ml. of a 1:20 mixture of concentrated nitric and sulfuric acids. Heat to 105° for 2 hours, cool, and pour into 50 ml. of water. Rinse with a further 50 ml. of water. Extract with 50 ml. of ether which has been freed of peroxides and aldehydes by shaking with water and then with 1:9 sulfuric acid saturated with ferrous sulfate. The ether used should give no reaction with potassium iodide solution.

Separate the aqueous layer and re-extract with 50 ml. of ether. Wash the first ether extract with 30 ml. of 2% sodium hydroxide solution and use this solution to extract the second ether extract. Discard the aqueous layer and extract the ether layers separately with 30-ml. portions of saturated sodium chloride solution. Combine the ether extracts and add 0.5 ml. of heavy mineral oil. Distil off the ether, evaporating off the last few drops with a stream of air.

Add 10 ml. of butanone which has been purified by distillation, using the 79-81° fraction. Add 1 ml. of 40% potassium hydroxide solution. Shake for 15 minutes and take absorbance measurements at 462 mμ and 565 mμ within 5 minutes.

As standards, prepare solutions of *m*-dinitrobenzene and 2,4,6-trinitrotoluene by dissolving 0.2 gram of each compound in concentrated nitric acid. Dilute an aliquot with concentrated nitric acid to give a solution containing 200 micrograms per ml. This may be kept as a stock solution.

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[18] M. Mirjolet and C. Domange, *Bull. soc. pharm Nancy* **No. 41**, 23-9 (1959).

[19] W. Hancock and E. Q. Laws, *Analyst* **80**, 665-74 (1955); **81**, 37-41 (1956).

Dilute 5 ml. of the stock solution to 100 ml. with concentrated sulfuric acid. This gives a solution containing 10 micrograms per ml. in nitration acid. Dilute appropriate amounts with nitration acid and treat as sample.

From the standard solutions the absorbance equivalent to 1 microgram of *m*-dinitrobenzene and 2,4,6-trinitrotoluene is found for the two wave lengths 462 mμ and 565 mμ. These will vary with different batches of butanone. Values obtained with one batch were as follows:

	462 mμ	565 mμ
m-dinitrobenzene	0.00080	0.01320
2,4,6-trinitrotoluene	0.00786	0.00437

If the absorbancies obtained with a mixture are a and b at the two wave lengths and the amounts of *m*-dinitrobenzene and 2,4,6-trinitrotoluene are x and y, then:

$$0.00080\,x + 0.00786\,y = a \qquad (462\ \text{m}\mu)$$
$$0.01320\,x + 0.00437\,y = b \qquad (565\ \text{m}\mu)$$

Solving for x and y we obtain:

$$x = 78.4\,b - 43.6\,a$$
$$y = 131.7\,a - 8.0\,b.$$

Substituting the numerical values for absorbancies for a and b we obtain the figures for *m*-dinitrobenzene and 2,4,6-trinitrotoluene.

Benzene and chlorobenzene. Mix 0.5 ml. of sample with 10 ml. of a mixture of equal parts of concentrated sulfuric acid and fuming nitric acid at 0°. After 1 hour dilute to 50 ml. with 1:1 concentrated sulfuric acid-concentrated nitric acid. Dilute 4 ml. of the mixture with 15 ml. of water and neutralize with 20% sodium hydroxide solution. Add 10 ml. of 10% sodium carbonate solution and reflux for 30 minutes. Cool and dilute to 50 ml. Extract 2 ml. with 20 ml. of butanone. Agitate 10 ml. of the butanone extract in the dark for 15 minutes with 5 ml. of 50% potassium hydroxide solution. Read at 570 mμ for benzene and at 420 mμ for chlorobenzene.

By potassium hydroxide in acetone-benzene solution.[20] *Air.* The preparation of the sample is not unlike that for development by butanone. Pass a volume of 0.2-0.5 liter of air at a velocity of 10 liters per hour through 2 ml. of a 1:20 mixture of concentrated nitric and sulfuric acids. When absorption is complete, heat at 110° for 45 minutes. Cool, dilute with 10 volumes of water, and cool. Extract by shaking with 6 ml. of benzene for 2 minutes. Mix 3 ml. of the benzene extract with 7 ml. of acetone and 1 ml. of 70% potassium hydroxide solution. Shake thoroughly and let stand in the dark for 15-30 minutes. Measure the absorbance at 460 mμ and 570 mμ. Illustrative details are given under the butanone technic.

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[20] F. I. Berezovskaya, B. E. Reznik, and S. S. Gitis, *Nauch. Zapiski, Dnepropetrovsk. Gosudarst. Univ.* 43, 45-52 (1953); J. Piotrowski, *Med. Pracy* 6, 149-58 (1955).

By pyridine and sodium hydroxide.[21] Prepare the sample by nitration and heating as described under the preceding methods. Extract with ether as for development with butanone. Develop the 10 ml. of the ether extract with 1 ml. of acetone and 0.5 ml of 4% solution of sodium hydroxide in ethanol. Agitate, let stand for 5 minutes, and add 0.5 ml. of 1:9 acetic acid containing 5% of pyridine. Mix well, let stand for 10 minutes, and read at 490 mμ. Calculate benzene and toluene as described in more detail under butanone as the reagent. The accuracy is to 0.001 mg. for benzene and to 0.01 mg. for toluene.

By nitrite and peroxide. [22] *Coke-oven gases.* Scrub the gas to remove naphthalene, hydrogen sulfide, ammonia, and carbon oxides. To an appropriate volume add 10 ml. of 1:1 acetic acid, 3-4 drops of 30% hydrogen peroxide, and 5 ml. of 10% sodium nitrite solution. After shaking for 10 minutes add 2 ml. of 40% sodium hydroxide solution. Shake for 15 minutes and set aside for 10 minutes before reading.

In the ultraviolet.[23] *Air.* Absorb the benzene by passing the air through ethanol chilled to 0°. By the use of proper equipment the ethanol will absorb up to 500 ppm. with an efficiency of 97-99%. Read the absorbance at 254.5 mμ or 248.5 mμ. Over the range of 0-500 ppm. the absorbancy is a straight line.

STYRENE

Styrene dissolved in choloroform absorbs in the ultraviolet while polystyrene does not (Vol. III, pp. 18-20). Direct reading in chloroform solution has been expanded to include styrene in polyester resins containing polymeric esters of dihydric alcohols and dibasic acids.[24] A correction is applied for fumerate and maleate esters present. By this method phthalate and glycols are also determined. Diallyl phthalate interferes with the phthalate determination. Triallyl cyanurate and diallyl phthalate should be separated.

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[21] Z. Peizker, *Pracovní lékařství* **4,** 358-65 (1952).

[22] F. P. Nikonyuk, *Kors i Khim.* **1956,** No. 2, 43-4.

[23] H. Kita, H. Maeda, T. Oshima, B. Hanazaki, and K. Hirasawa, *Bull. Tokyo Med. Dental Univ.* **2,** 287-90 (1956).

[24] R. C. Hirt, R. G. Schmitt, and R. W. Stafford, *Anal. Chem.* **27,** 354-6 (1955).

Procedure—Dissolve the sample in chloroform. Read at 282 mμ and 291 mμ and calculate the styrene and phthalate from the following equations:

$$A_{291} = bc_s\,(5.58\,C_m + 1.39\,C_p)$$
$$A_{282} = bc_s\,(8.18\,C_m + 6.85\,C_p)$$

or from the following absorbance ratio:

$$\frac{A_{282}}{A_{291}} = \frac{8.18\,C_m + 6.85\,C_p}{5.58\,C_m + 1.39\,C_p}$$

where A is the observed absorbance at the indicated wave length
b is the cell light path in millimeters
c_s is the sample concentration in grams per 100 ml.
C_m and C_p are fractions or % concentrations of the monomeric styrene and phthalate component of the polyester, respectively.

Estimate the fumerate concentration from the following formula:

$$C_f = (1/\beta)(100 - C_m - \alpha C_p)$$

where C_f is the fraction or per cent concentration of fumerate
α is 1 FW_g/FW_p
β is 1 FW_g/FW_f
FW_g is the formula weight of the glycol
FW_p is the formula weight of the phthalate
FW_f is the formula weight of fumarate.

Values for α and β for commonly encountered glycols are as follows:

Glycol	α	β
Propylene	1.256	1.369
Dipropylene	1.610	1.877
Ethylene	1.171	1.246
Diethylene	1.439	1.631

Calculate the absorbance contributed by fumarate from the following equation:

$$A_f = a_f \times b \times c_s \times C_f.$$

Recalculate the styrene and phthalate concentrations taking these corrections into consideration. Re-estimate the fumarate concentration and estimate the concentration of the glycol residue by subtracting from 100%.

POLYSTYRENE

The absorbancy of polystyrene at 269 mμ is a basis for determining both copolymers and true homopolymers since it is a measurement of the number of benzene (phenyl) rings present regardless of the exact composition of the polymer chain.[25] The absorption of diene fatty acid over the range 260-300 mμ is so low that it may be ignored. Amounts of conjugated tetraene acids in commercial fatty acids and oils are negligible. Corrections must be made for monomeric styrene, which absorbs at 291 mμ, and for conjugated triene acids, at 282 mμ. The correction for conjugated triene acids is effective up to about 6% of acid in the presence of about 20% of polymerized styrene. Styrenated tung oil acids cannot be analyzed by this method since they contain a high concentration of conjugated triene acids. To overcome this, the sample may be halogenated or hydrogenated to destroy the conjugated unsaturation.

Sample—*Styrenated fatty acids.* Dissolve a weighed sample in cyclohexane.

Alkyd resins.[26] To an appropriate sample add 150 ml. of benzene and warm to dissolve if necessary. Add 60 ml. of 6.6% alcoholic potassium hydroxide and connect with a reflux. After 1 hour at 40° slowly heat to boiling and reflux for 90 minutes. Rinse down the condenser with 1:3 absolute ethanol-benzene. Replace the reflux with a soda-lime guard tube and cool.

After cooling filter as rapidly as possible through fritted glass, using the alcohol-benzene solution for transfer and washing the reaction flask. After about 75 ml. of wash solution has passed through the filter, check to see that the washings are now not alkaline to phenolphthalein. If necessary wash further.

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[25] R. C. Hirt, R. W. Stafford, F. T. King, and R. G. Schmitt, *Ibid.* 27, 226-8 (1955).

[26] ASTM Standards 1955, Vol. 8, p. 354.

Neutralize the anhydrous filtrate by slow addition of concentrated hydrochloric acid. Cool and filter off the precipitate of potassium chloride. Concentrate the filtrate to a manageable volume and extract with ethyl ether. The extract contains the polystyrene, fatty acids, and reaction products. Evaporate to dryness and dissolve the residue in cyclohexane. Dilute to a known volume.

Procedure—Read the cyclohexane solution at 291 mμ, 282 mμ, and 269 mμ. Calculate the concentration of polystyrene from the following formula:

$$C_p = \frac{0.63673}{c_s b}(A_{269} - 1.293\,A_{282} + 0.207\,A_{291})$$

where c_s is the concentration of the sample in solution in grams per 100 ml.
b is the cell length in millimeters.

If the A_{291} reading becomes appreciable in respect to the A_{269} and A_{282} readings, a high concentration of residual monomeric styrene is present and the amount of this monomer must be calculated by the following formula:

$$C_m = 0.00967\left[\frac{1}{c_s b}(19.64\,A_{291} - A_{282}) - 0.310\,C_p\right].$$

The individual components are determined by the following simultaneous equations:

monomeric styrene

$$A_{291} = c_s b\,(0.0175\,C_p + 8.50\,C_t + 5.64\,C_m)$$

conjugated triene acids

$$A_{282} = c_s b\,(0.0342\,C_p + 166.9\,C_t + 7.30\,C_m)$$

polystyrene

$$A_{269} = c_s b\,(1.61\,C_p + 214\,C_t + 8.27\,C_m)$$

where p, t, and m represent polymerized styrene, conjugated triene acids, and monomeric styrene respectively.

Diphenyl, Biphenyl

Reading of biphenyl in the ultraviolet (Vol. III, p. 22) when contaminated with citrus oil requires a correction for the citrus oil.[27] Since the absorption varies with different fruits, this is open to question. The reading of the extracted citrus oil in the infrared at 13.90 μ has also been used [28] (Vol. III, p. 22). Chromatographic separation from the citrus oil permits direct reading at 248 mμ.[29] This recovers from 0.1 ppm. in juice up to 600 ppm. in fruit peel.

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[27] A. P. Steyn and F. Rosselet, *Analyst* **74**, 89-95 (1949); Cf. Karl Erik Almin, *Svensk Papperstidn.* **59**, 44-50 (1956).

Difficulties which arise when biphenyl is determined in citrus fruits from the loss of biphenyl during removal of essential oils are overcome if the essential oils are removed with 6% potassium permanganate solution in glacial acetic acid. The sensitivity of the ultraviolet absorption method is increased by converting the biphenyl to the sulfonic acid so that the absorption can be measured in water rather than an alcohol.[30]

Nitration of biphenyl in acetic acid solution gives 4-nitrobiphenyl. When reduced to the amine and diazotized this couples with *N*-(1-naphthyl)ethylenediamine to give a purple color with a maximum absorption at 570 mμ.[31] Beer's law is followed for 0.01 to 0.1 mg. of biphenyl.

Sample—*Citrus juice.* Place a 1500-ml. sample in a 3-liter boiling flask fitted with a 10-ml. lighter-than-water receiving trap. Place one milliliter of *n*-heptane or isooctane in the receiver and boil the flask gently for 3 hours. Rinse the condenser and still head with 1 ml. of the solvent, read the total volume of *n*-heptane or isooctane containing citrus oil and the biphenyl, and drain into a flask.

Prepare chromostrips by blending 19 grams of reagent-grade silicic acid, 1 gram of Clinco-15 modified starch, or a 2 to 1 mixture of cornstarch and tapioca flour, 0.15 gram of zinc silicate, and 0.15 gram of zinc cadmium sulfide. Mix with 36 ml. of water. Heat at 85° and stir constantly until the mixture thickens, stirring an additional 30 seconds at this temperature. Add 2-7 ml. of water, stir, and spread on glass strips 36 × 136 mm. Hold the glass between glass guides 0.02 inch higher than the glass strip. Dry in a forced draft oven at 105° for 15 minutes. Place the strips in a desiccator over powdered potassium hydroxide and evacuate to 3 mm. of mercury. Use a tube packed with Ascarite to admit dry air when the strips have reached equilibrium. The strips are not usable after exposure to the atmosphere for periods longer than 10 minutes.[32] Wash the strips by placing in stoppered test tubes containing 1 ml. of 95% ethanol and allowing the solvent to travel up the strip. Dry at 85° for 4 minutes in an oven and cool in a desiccator. Doing this just prior to use of the strips removes impurities absorbed from the air and therefore decreases the blank.

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[28] E. J. Elvin and L. R. Knodel, *Anal. Chem.* **26**, 1234-6 (1954).

[29] J. G. Kirchner, J. M. Miller, and R. G. Rice, *J. Agr. Food Chem.* **2**, 1031-3 (1954); W. L. Stanley, S. H. Vannier, and B. Gentili, *J. Assoc. Offic. Agr. Chemists* **40**, 282-6 (1957).

[30] Bernard Koether, *Z. Lebensm.-Untersuch. u. -Forsch.* **108**, 158-63 (1958).

[31] R. B. Bruce and J. W. Howard, *Anal. Chem.* **28**, 1973-5 (1956).

[32] J. G. Kirchner, J. M. Miller, and G. J. Keller, *Ibid.* **23**, 420-5 (1951).

Apply the biphenyl solution containing 0.002-0.011 mg. of biphenyl to the strips by means of a syringe microburet capable of delivering 0.1 microliter of solution. Place in test tubes containing 1 ml. of hexane, free from ultraviolet-absorbing impurities. Stopper and allow the solvent to proceed up the strip by capillarity to 10 cm. above the origin. This should require about 15 minutes and gives good separation. The oxygenated constituents of citrus oil are tightly sorbed at the origin.[33]

Dry the strips in the air stream until the solvent odor disappears. Determine the position of the biphenyl on the strip under ultraviolet light and transfer these spots to a sintered glass funnel by scraping off a standard area 22 mm. long the full width of the strip with the biphenyl spot centered. Elute the biphenyl with 95% ethanol and dilute to 3 ml. This sample solution is then ready for direct reading.

Citrus fruit. Grind a single fruit with 200 ml. of water in a blender to a slurry and dilute to 1500 ml. with water. Treat as described for citrus juice.

For reading in aqueous solution grind a 200-gram sample in 200 ml. of water. Steam-distil the biphenyl and dissolve in petroleum ether. Add 100 ml. of 6% potassium permanganate solution in glacial acetic acid. After 5 minutes steam-distil again and dissolve [illegible] petroleum ether. Evaporate the solvent to remove traces of essential oils. Treat the residue with 1 ml. of concentrated sulfuric acid for 10 minutes at 105°. Cool and read directly.

Citrus peel. Grind 100 grams of peel to a slurry with 100 ml. of water and dilute to 1500 ml. Treat as described for citrus juice.

Blood. Shake 5 ml. of oxalated blood for 2-3 minutes with 10 ml. of chloroform and filter through chloroform-wetted glass wool. Wash the precipitate and tube with chloroform. Shake the chloroform layer with 10 ml. of 20% sodium hydroxide solution for 2-3 minutes to remove phenols. Draw off the aqueous layer, wash the chloroform twice with 10-ml. portions of water, and complete the washing by shaking mechanically for 1 hour with a mixture of 10 ml. of 6% potassium permanganate solution and 5 ml. of glacial acetic acid. Draw off the permanganate layer and shake the chloroform layer with 20 ml. of 5% sodium carbonate solution for 2 minutes. Remove the carbonate layer and wash the chloroform twice with 20 ml. of water. Remove the water, filter the chloroform through chloroform-wetted glass wool into a 22 × 200 mm. test tube, and rinse with chloroform.

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[33] J. G. Kirchner and J. M. Miller, *Ind. Eng. Chem.* **44**, 318-21 (1952).

Urine. Shake a 5-ml. sample for 2-3 minutes with 10-ml. of chloroform. Separate the urine layer and discard. Wash the chloroform layer with water and discard the washings. Treat as for blood starting at "Shake the chloroform layer with 10 ml. of 20% sodium hydroxide. . . ."

Feces. Mix a 5-ml. sample in a mortar with anhydrous sodium sulfate until the sample is dry. Transfer to a Soxhlet thimble, extract with chloroform for 3 hours, and dilute the extract to 100 ml. with chloroform. Treat an aliquot as described for blood starting at "Shake the chloroform layer with 10 ml. of 20% sodium hydroxide. . . ."

Procedure—*Direct reading.* The prepared sample is in ethanol or water solution. Read at 248 mμ for ethanol solution or at 263 mμ in water and correct for a blank obtained daily.

By N-*(1-naphthyl)ethylenediamine.* The sample of 0.01-0.08 mg. is in chloroform in a 22 × 200 mm. test tube. Add 0.5 ml. of glacial acetic acid to prevent loss of biphenyl during evaporation of chloroform, and evaporate the chloroform to 1-2 ml. at 50-55° under a stream of air. Add 0.5 ml. of concentrated nitric acid and heat at 70° for one hour. Stop the nitration by adding 5 ml. of ice water. Add 2 ml. of chloroform and extract the nitro derivative by shaking the tube for three minutes. Draw off the acid layer and discard. Evaporate the chloroform under a gentle stream of air at 50-60°. Add 1 ml. of 95% ethanol and 2 ml. of 1:1.5 hydrochloric acid. Shake well to wash down the sides of the tube. Add 0.2 gram of zinc dust, fit with a small finger condenser, and place in boiling water for 15 minutes. Filter through paper. Add rinsings of the condenser and tube to a total of about 40 ml. Add 1 ml. of 0.25% sodium nitrite solution, mix, and allow to stand for 10 minutes. Add 1 ml. of 2.5% ammonium sulfamate solution and allow to stand for 10 minutes. Add 2.0 ml. of 1% aqueous *N*-(1-naphthyl)ethylenediamine dihydrochloride solution, dilute to 50 ml., mix, and allow to stand for one hour. Read the absorbance at 570 mμ against a blank.

INDENE

The reaction to produce fulvenes (Vol. III, pp. 25-6) has been somewhat modified.[34]

Procedure—As sample use 10 ml. of toluene solution containing 1-10% of indene. Add 5 ml. of 20% benzaldehyde solution in ethanol and 5 ml. of 5% potassium hydroxide solution in ethanol. Shake well and let stand for 30 minutes from the time the potassium hydroxide was added. Decant the hydrocarbon layer into a tube containing 1 gram of sodium sulfate, shake, and read against 10 ml. of 1:14 acetic acid carried through the same procedure.

BENZO[*a*]PYRENE

The carcenogenic properties of benzo[*a*]pyrene has led to its investigation and separation in American shale oil.[35] The somewhat complex technic (Figure 2) involves the following steps: (1) vacuum separation into 3 distillates and a residue; (2) chromatographic fractionation in cyclohexane on Florasil or (2a) alternatively in dichloromethane on activated alumina; (3) chromatographic fractionation on silica gel to give a benzene eluate; and (4) chromotographic fractionation in cyclohexane on activated alumina to give a cyclohexane-benzene eluate.

Sample—*Shale oil. Fractionation.* (1) Assemble an all-glass distilling apparatus using a jacketed heated column of only a few theoretical plates and a low holdup rather than a more efficient column with considerable holdup. Connect the side arm to the column by an inner seal to avoid the creeping over of material. Distil a 10-30 gram sample *in vacuo,* stirring by means of a magnetic stirrer, at the rate of about 2 mg. of distillate per minute per gram of charge. Use a water pump until the bath temperature reaches 90-100° and then replace with an oil pump, slowly decreasing the pressure to 30-40 microns.

Temperatures are expressed as converted to 760 mm. Collect the fraction distilling between 400° and 510° separately. Stop the distillation when the boiling point reaches 510°. The bath temperature should not exceed 250° to prevent any pyrolytic formation of benzo[*a*]pyrene.

Purify the solvents to remove fluorescent substances. Reflux methanol

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[34] W. Roman and M. Smith, *Analyst* 78, 680-1 (1953).

[35] H. J. Cahnmann, *Anal. Chem.* 27, 1235-9 (1955).

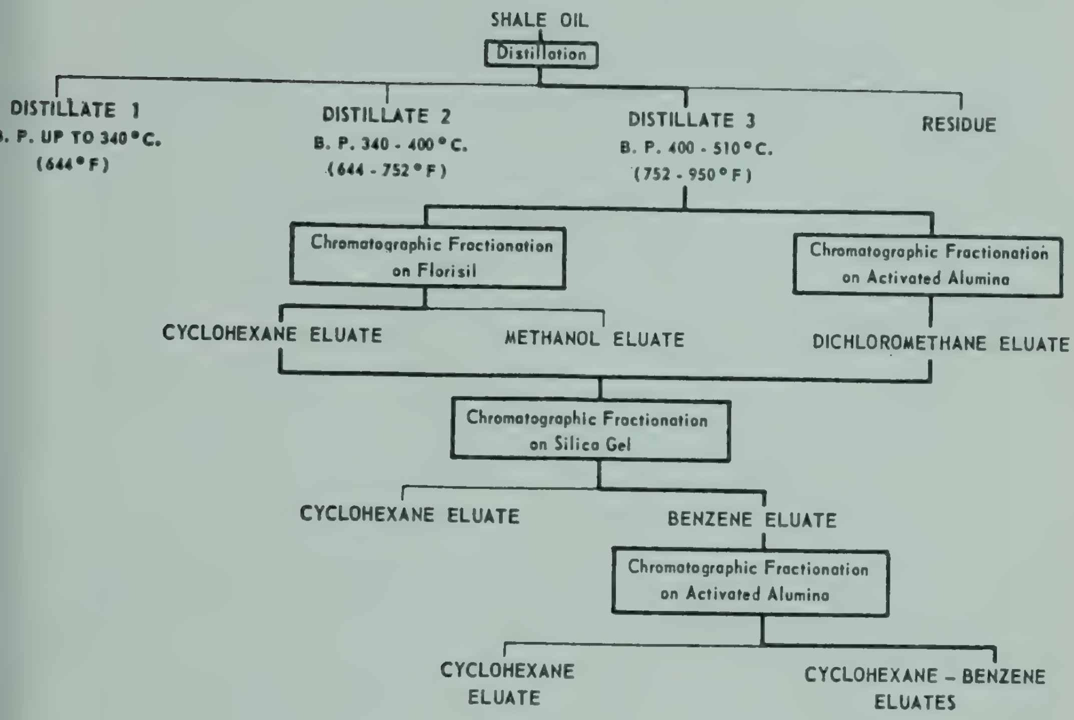
SHALE OIL
Distillation
DISTILLATE 1
. P. UP TO 340°C.
(644°F)
DISTILLATE 2
B. P. 340 - 400°C.
(644 - 752°F)
DISTILLATE 3
B. P. 400 - 510°C.
(752 - 950°F)
RESIDUE
Chromatographic Fractionation on Florisil
Chromatographic Fractionation on Activated Alumina
CYCLOHEXANE ELUATE
METHANOL ELUATE
DICHLOROMETHANE ELUATE
Chromatographic Fractionation on Silica Gel
CYCLOHEXANE ELUATE
BENZENE ELUATE
Chromatographic Fractionation on Activated Alumina
CYCLOHEXANE ELUATE
CYCLOHEXANE – BENZENE ELUATES

FIG. 2

Shale oil fractionation

over freshly precipitated silver oxide and distil. Shake all other solvents with activated carbon and distil. In each case discard the first and last 10% of the distillate.

Fractionation. (2) Prepare a chromatographic column of 30-60 mesh Florisil, a magnesium trisilicate. Dissolve the 400-510° fraction in 1-5 volumes of cyclohexane and pass it through the column at the rate of 5-15 mm. per minute, a rate also used in development of the chromatograph.

Develop by passing cyclohexane through the column and observe under ultraviolet light. When the strongly blue-purple zone surrounding a pale yellow zone approaches the end of the column, change receivers. After the effluent reaches the same refractive index as cyclohexane, change the developing solvent to methanol but do not change the receiver.

The column observed in the daylight will now develop as follows: a light purplish-gray zone at the top, then a light brown zone, then a dark purple line or narrow zone, and finally a brown zone. As soon as the narrow dark purple line is about to leave the column, and the effluent changes from yellow to dark brown, change the receiver.

Alternative fractionation. (2a) Alternatively, pass an aliquot of the distillate, dissolved in dichloromethane, through an activated alumina column and develop with dichloromethane. Observed under ultraviolet light, a broad purplish zone appears at the top, then a yellow weakly fluorescent zone, and finally a narrow blue fluorescent zone. When the blue zone reaches the bottom, change receivers. After the purplish zone is collected and the refractive index of the effluent is the same as that of dichloromethane, discontinue collecting even though the effluent still shows a blue fluorescence.

Fractionation. (3) Concentrate the cyclohexane eluate (2) to a small volume under reduced pressure or evaporate the dichloromethane eluate (2a) completely and take up in 1-5 volumes of cyclohexane. Chromatograph the solution on activated alumina and develop with cyclohexane. When the effluent turns from yellow to brown to colorless and returns to its original refractive index, change the developer to benzene. At this point the benzo[*a*]pyrene is in the column. The column should show a narrow pale yellow zone, then a broad purplish zone, when observed under ultraviolet light. When the pale yellow zone is a short distance from the end of the column, change the receiver. Stop collecting when the bulk of the purplish zone has left the column and the refractive index of the effluent is the same as that of benzene. It is frequently desirable to follow the course of the benzo[*a*]pyrene spectrophotometrically. In this case, collect the benzene eluate in a number of small fractions and combine those fractions which show evidence of benzopyrene and work up further.

Fractionation. (4) Evaporate the benzene eluate under reduced pressure and take up 1-5 volumes of cyclohexane. Chromotograph again on activated alumina. Develop with cyclohexane. After the refractive index of the effluent becomes the same as that of cyclohexane, continue the elution with cyclohexane-benzene mixtures of gradually increased benzene content. Observe the several zones under ultraviolet light—the one containing the bulk of the benzo[*a*]pyrene is pale yellow and weakly fluorescent. This zone leaves the column when the cyclohexane benzene mixture contains 40-60% benzene. Determine when the elution is complete with fluorescence spectra of the various eluate fractions.

Procedure—Read at 403 mμ. Although the benzo[*a*]pyrene peaks are higher at 384 mμ and 364 mμ, 403 mμ is chosen for reading because there is less background absorption in this region.

NAPHTHALENE

Naphthalene in the concentration of 0.2 to 200 ppm. is determined by a reaction with anhydrous aluminum chloride in chloroform solution with an accuracy of ±5.6%.[36] The color formed varies from light to deep blue depending on the naphthalene concentration. Although the colored solution may be read at 545 mμ with a spectrophotometer, the maximum absorbance is obtained at 525 mμ and there is no measurable change in the readings from slightly varied colors. The presence of water in the chloroform solution inhibits color formation as hydrochloric acid is formed by the reaction of water on aluminum chloride. Phenol does not effect the reaction when a 1% sodium hydroxide solution is added to prevent extraction of phenol by the chloroform. Interfering sulfides are removed by addition of silver nitrate and sodium hydroxide. Anthracene does not interfere.

Naphthalene and naphthoquinone are read directly in methanol. The technic is one developed primarily for analysis of phthalic anhydride.[37] The procedure is carried out under red light as the solutions are light-sensitive. Maleic anhydride and benzoic acid do not interfere. The error in the determination of naphthalene in phthalic anhydride depends on its ratio to naphthoquinone. In proportions by weight of 10:1 or higher, the relative error averages ±1% while in proportions of 1:1 the relative error increases to ±3 to 5%.

If the percentage of naphthalene is small, higher aromatic hydrocarbons such as anthracene and phenanthrene may interfere. It is possible to determine if the naphthalene reading is actually from these compounds by measuring the solution after 17 hours at 309.5 mμ. When naphthalene is present, there is a distinct decrease in the absorbance appropriate to the absorption minimum.

Naphthalene gives a turbidity of naphthalene picrate which is a reliable method of determination.[38] A high concentration of picric acid reduces the solubility and increases the sensitivity. This is applied to determine naphthalene in gas.

Sample—*Industrial wastes. In the presence of sulfides.* Add 10 ml of 10% silver nitrate solution to an appropriate sample. Filter and add 10 ml. of 10% sodium hydroxide solution. Filter and shake with 5 ml. of chloroform for 1 minute. Allow 1 minute for separation of the layers and drain the chloroform layer into a cylinder containing 1 ml. of 80- to 200-mesh alumina as dehydrating agent, admitting as little water as possible. Add 5 ml. of chloroform to the sodium hydroxide layer and repeat the extraction. Shake the combined chloroform extracts and alumina for 1 minute and allow 1 minute for the alumina to settle. Filter 7 ml. of the chloroform solution containing naphthalene and develop with aluminum chloride.

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[36] A. L. Medin and L. K. Herndon, *Sewage and Industrial Wastes* 24, 1260-5 (1952).

[37] Hans Peters, *Anal. Chem.* 31, 1326-31 (1959).

[38] L. J. Kane, J. J. Gentile, K. E. Trostle, S. Katell, and C. C. Shale, *U. S. Bur. Mines, Repts. Invest.* No. 5368, 12 pp. (1957).

In the absence of sulfides. Shake a filtered sample with 5 ml. of 1% sodium hydroxide solution and follow the technic described in the presence of sulfides starting at ". . . shake with 5 ml. of chloroform for 1 minute."

Procedure—*By aluminum chloride.* To 7 ml. of the chloroform extract containing 0.2 to 200 ppm. of naphthalene, add 0.4 gram of anhydrous aluminum chloride and stopper. Shake for 2-3 minutes, remove the stopper, and let stand for exactly 3 minutes. Centrifuge a 5-6 ml. portion for 1 minute and read at 545 mμ or at 525 mμ.

Phthalic anhydride. Store the sample in the dark and weigh under red light. Dissolve 1-5 grams in methanol and dilute to 100 ml. with methanol. Dilute a 10-ml. aliquot to 100 ml. with methanol. Read at 347 mμ for 1,4-naphthoquinone. Store the remaining solution for 17 hours without precautions and read at 311 mμ for naphthalene.

$$\text{Naphthoquinone} = A_{347}/14.79$$

$$\text{Naphthalene} = [A_{311} - (12.45 \times C_{\text{NQ}}) - (0.0015 \times C_{\text{PA}}]/1.95.$$

PA stands for phthalic anhydride as separately determined, page 400.

By picric acid. As reagent heat about 7 grams of picric acid to 50° in 1 liter of water. Filter hot and let cool. The clear solution should have an optical density under 0.08 at 600 mμ.

Pass 3 cubic feet of filtered gas at 2 cubic feet per hour through 50 ml. of the reagent at 3-4°. There should be large crystals of picric acid present which settle readily. Add 0.1 ml. of Santomerse *S*, stopper, and heat at 35° for 5 minutes. Cool to 25° and read at 600 mμ. The calibration curve should show no turbidity up to 0.25 mg. of naphthalene.

AZULENE

The blue color in organic solvent (Vol. III, p. 26) has received further study.[39] Suitable solvents are xylol or a mixture of *p*-dibromobenzene, bromobenzene, and *o*-dichlorobenzene for reading at 560 mμ.

Sample—*Camomile.* Mix 10 grams of sample with 400 ml. of saturated salt solution. Steam-distil for 8 hours. After 5 minutes take up the distilled oil in 0.8 ml. of xylol. Separate and dilute to 6 ml. with the solvent.

Extracted dust. Mix 10 grams of sample with 400 ml. of saturated salt solution. Add 2 ml. of a mixture of equal parts of *p*-dibromobenzene, *o*-dichlorobenzene, and bromobenzene. Steam-distil for 8 hours. Separate the distilled oil and solvent and dilute to 6 ml. with the same solvent.

Procedure—Read the solution at 560 mμ.

CAROTENOIDS

The family of carotenoids includes carotene, xanthophylls, and to a lesser extent other red and yellow pigments. One may read the total carotenoids at 436 mμ, separate the major ingredient, carotene, usually β-carotene, and similarly read it. The methods of isolation show a definite trend from direct extraction (Vol. III, pp. 27-37) toward chromatography. What this all leads to then is carotene in solution in a diversity of solvents.

The isolation by chromatography is usually feasible. However, if the sample is alfalfa a difficulty may develop since *N,N′*-diphenyl-p-phenylene-diamine and other stabilizers or antioxidants added to dehydrated alfalfa cause a yellow color with the magnesia sorbant often used for chromatography.[40] Both tricalcium phosphate, which also sorbs chlorophyll,[41] and aluminum oxide[42] have been used as sorbants to overcome this interference. If 5 ml. of 0.2% stannous chloride solution in absolute ethanol is added to the eluate following chromatography, the interfering yellow color is prevented.[43] The presence of 1-5% of lard in alfalfa causes greater error but not sufficient to justify introducing a saponification step.[44]

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[39] R. Fischer and H. Resch, *Pharm. Zentralhalle* **91**, 265-70 (1952).

[40] R. E. Beachene and H. L. Mitchell, *J. Agr. Food Chem.* **1**, 461-4 (1953).

[41] H. L. Mitchell and R. E. Silker, *Ibid.* **1**, 1163-5 (1953).

[42] V. H. Booth, *Analyst* **79**, 507-9 (1954).

[43] Harold A. Kaler, *J. Agr. Food Chem.* **4**, 70-1 (1956).

Although azobenzene and potassium dichromate are often utilized as standards for β-carotene, a need arose for the development of a stable β-carotene solution for interlaboratory collaborative studies. β-carotene in mineral oil, which is purified and degassed by passing through a 75 × 250 mm. alumina column under reduced pressure, is stable for 9 months in the range of concentration suitable for spectrophotometric determinations, and compares favorably with carotene standards prepared in 10% acetone solution in hexane.[45]

Only if the sunlight falls directly on samples left unprotected is photochemical destruction appreciable. Therefore under normal laboratory conditions destruction by sunlight is negligible.[46] Carotene-destroying enzymes are inactivated by blanching, autoclaving, or drying, and the carotene extracted from the dried material, carotene being more easily extracted from blanched than unblanched samples.

The formation of a bluish-green color when perchloric acid [47] is added to carotene permits the determination either separately or simultaneously with vitamin A. In the simultaneous determination with vitamin A, amyl acetate is used to stabilize the color. The colors must be read within 15 minutes after the addition of the perchloric acid since the vitamin A purplish-red color fades to yellow after 30 minutes. Beer's law is followed.

Activation of Alumina[48]—The technic which follows provides the necessary activation for this sorbant. Add 0.5 kg. of alumina to 1.5 liters of 1:35 sulfuric acid and heat at 60-70° for 15 minutes, stirring constantly. Wash 3 times with hot water until neutral. Repeat the heating with sulfuric acid and washing 3 times, with a final washing with hot water until neutral. Treat the alumina 3 times with 2% sodium hydroxide solution at 60-70° for 15 minutes and wash with hot water until neutral. Filter and heat at 305-375° for 2 hours.

Separation of Carotene from Total Carotenoids.[49]—Apply suction to an 18 × 240 mm. absorption column loosely plugged with cotton. Add a 1:1 mixture of activated magnesia and diatomaceous earth in small amounts to a height of approximately 11 cm., press down once, and place 1-2 cm. anhydrous sodium sulfate on top. Transfer an aliquot of sample solution containing carotene in petroleum ether to a distillation flask and concentrate to 5 ml. with suction continuously applied. Add to the column and rinse with four 5-ml. portions of petroleum ether to remove all color. Rinse down the sides of the column with a few ml. of petroleum ether. After a few drops have come through the column change to another suction flask. When nearly all of the petroleum ether is down to the sodium sulfate layer, add 50 ml. of a 9:1 mixture of petroleum ether and acetone. Carotene passes rapidly through the column and can be observed visually. Bands of xanthophylls and of chlorophylls and carotene oxidation products may be visible in the column. Collect the carotene eluate and dilute to 100 ml. with 1:9 acetone-petroleum ether for reading at 436 mμ.

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[44] F. W. Quackenbush, *J. Assoc. Offic. Agr. Chemists* **39**, 139-41 (1956).

[45] W. J. Rabourn and E. D. Schall, *Anal. Chem.* **28**, 1197-8 (1956).

[46] H. L. Mitchell and H. H. King, *Ibid.* **20**, 637-8 (1948).

[47] Peter Flesch, *Proc. Soc. Exptl. Biol. and Med.* **84**, 148-9 (1953).

[48] Ryichiro Ishii, *J. Fermentation Technol. (Japan)* **29**, 254-6 (1951).

[49] "Official Methods of Analysis of the Association of Official Agricultural Chemists," 8th ed., pp. 235, 816 (1955), Association of Official Agricultural Chemists, Washington, D. C. Cf. A. Inczèdy and E. Demel, *Élelmezési Ipar* **10**, 221-5 (1956).

Samples—*Hard-wheat meal and alimentary-dough products.*[50] Extract a 10-gram finely ground sample with 25- to 30-ml. portions of butanol saturated with water until 100 ml. of filtrate is obtained. Filter and clear turbid solutions by standing at 0-1°. Read directly.

Plant Material.[51] Grind 0.2-0.3 gram of dried sample, or 1-2 gram of fresh sample mixed with powdered glass with a 1:1 mixture of petroleum ether and methanol. Filter, add water, and wash the aqueous methanol layer with petroleum ether. Saponify the ether extracts 3-4 hours at 40° with an equal volume of an alcoholic potassium hydroxide solution containing 5 grams of potassium hydroxide, 5 ml. of water, and 90 ml. of methanol. If the layers separate at the beginning of saponification, add additional methanol. Add water and extract the aqueous methanol layer with petroleum ether. To free the petroleum ether extracts of xanthophyll wash with 90% methanol and remove the methanol by washing with water. Dry the solution containing α-carotene and lycopene with calcium chloride, concentrate to 20 ml. in an atmosphere of nitrogen, and pass through a 5 × 8 cm. column of aluminum oxide. A narrow greenish-yellow band of α-carotene is followed through the column by a wider band of β-carotene and a red band of lycopene. Determine α- and β-carotene separately or together in the eluate.

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[50] Manlio Brogioni, *Ann. fals. et fraudes* 50, 65-72 (1957).

[51] Hellmut Aust, *Mikhwiss Ber.* 5, 31-42 (1955).

Fresh plant materials and silages.[52] If the analysis cannot be performed immediately, blanch the finely cut sample in boiling water 5-10 minutes and store frozen. Blend a 2-5 gram sample with 40 ml. of acetone, 60 ml. of hexane, and 0.1 gram of magnesium carbonate for 5 minutes. Filter with suction or allow to settle, decant, and wash the residue with two 25-ml. portions of acetone followed by 25 ml. of hexane. Combine the extracts. Wash the acetone from the extract with five 100-ml. portions of water. Transfer the upper layer to a flask containing 9 ml. of acetone and dilute to 100 ml. with hexane. Ethanol may be substituted for acetone. In this case, substitute 80 ml. of 95% ethanol for 40 ml. of acetone in the blender step. Pass through a column of activated magnesia and diatomaceous earth following the procedure under "Separation of Carotene from Total Carotenoids." Read directly.

Alfalfa.[53] Wrap appropriate samples in towels and autoclave for 5 minutes at 5 pounds pressure to inactivate the enzymes which will otherwise reduce the carotene content. Dry for 4 hours at 65°. Grind to pass through a 20-mesh screen and take a 1-gram sample. Add 60 ml. of 33% acetone solution in petroleum ether boiling at 63-69°. Let stand in the dark overnight, filter, and wash with the petroleum ether using suction. Concentrate at 100° to about 40 ml. Pass through a column containing a 1:2 mixture of magnesia and Super-Cel. Elute the carotene with 4% acetone solution in the petroleum ether and read the carotene directly at 436 mμ.

Hay and dried plants.[54] Add 30 ml. of 30% acetone solution in hexane to a 2-gram, 40-mesh sample. If the carotene content is high use a 1-gram sample, a 4-gram sample if the content is low. Reflux for 1 hour at the rate of 1-3 drops per second and cool to room temperature. Alternatively, stopper and place in a dark room at room temperature at least 15 hours. Decant or filter, wash the residue, and dilute to 100 ml. with hexane.

As a chromatograph column prepare a 1:1 mixture of activated magnesia and diatomaceous earth. Place glass wool at the bottom of the column and add 15 cm. of sorbant. Apply vacuum and pack to 10 cm. Add

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[52] F. W. Quackenbush, *J. Assoc. Offic. Agr. Chem.* 38, 694-5 (1955); "Official Methods of Analysis of the Association of Official Agricultural Chemists," 8th ed., p. 816, Association of Official Agricultural Chemists, Washington, D. C. (1955).

[53] H. L. Mitchell and H. H. King, *Anal. Chem.* 20, 637-8 (1948); R. E. Silker, W. A. Schrenk, and H. H. King, *Ind. Eng. Chem., Anal. Ed.* 16, 513-5 (1944).

[54] F. W. Quackenbush, *J. Assoc. Offic. Agr. Chemists* 33, 650-1 (1950); "Official Methods of Analysis of the Association of Official Agricultural Chemists," 8th ed., p. 816, Association of Official Agricultural Chemists, Washington, D. C. (1955).

1 cm. of anhydrous sodium sulfate as an upper layer. Under vacuum pass the extract through. Keeping the top of the column covered with liquid, follow with 1:9 acetone-hexane to develop the chromatogram. Xanthophyll, chlorophyll, and oxidation products of carotene are retained in the column. Dilute the carotene eluate to 100 ml. with 1:9 acetone-hexane and read directly.

Eggs.[55] Homogenize the sample in an atmosphere of carbon dioxide. To an 80-gram portion, add 80 ml. of absolute methanol and 20 ml. of saturated aqueous potassium hydroxide solution. Heat in boiling water for 5 minutes with stirring and cool to room temperature. Take up in 150 ml. of peroxide-free diethyl ether and shake with 200 ml. of water. Separate the ether layer and extract the aqueous layer twice with 120-ml. portions of ether, followed by 4 times with 100-ml. portions of ether. The last extracts should be colorless. Wash the combined ether extracts with five 100-ml. portions of water. Dry with 80 grams of sodium sulfate and evaporate to dryness under reduced pressure at room temperature. Add a small amount of petroleum ether and repeat the evaporation to remove all diethyl ether. Dissolve the residue in hexane and dilute to 100 ml. with hexane.

For the chromatographing, pack a 17 × 2 cm. tube with analytical grade calcium carbonate and place a thin layer of sodium sulfate on top. Add 10 ml. of the sample in hexane. Chromatograph with 50 ml. of hexane followed with hexane containing 5% of acetone until the strong yellow zone of lutein moves to the bottom of the column. The eluate contains Vitamin A.

A faint pink band of zeaxanthin may be close above the lutein band. Separate the portion of the column containing these bands from the yellow zone of oxidation products higher up in the column. Extract the pigments from the sorbant with peroxide-free diethyl ether. Evaporate to dryness under reduced pressure and dissolve in petroleum ether, boiling at 80-95°. Read carotene directly.

Vitaminized chocolate.[56] Heat 1.5 gram of pulverized sample with 5 ml. of water at 40°. Transfer to a blender and wash the flask with 50 ml. of petroleum ether and 75 ml. of 95% ethanol, adding the washings to the blender. Homogenize for 30-60 seconds. Separate the ether layer and wash with petroleum ether until a clear layer results. Wash the ether extracts twice with 50 ml. of water. Read the carotene directly.

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[55] Stig Kyrning, *Intern. Z. Vitaminforsch.* **24**, 263-71 (1952).

[56] S. Berger and B. Dietl, *Roczniki Państwowego Zakładu Hig.* **6**, 181-4 (1955).

Butter.[57] Reflux a 5-gram sample with 40 ml. of 95% ethanol and 50 ml. of 50% potassium hydroxide solution for 30 minutes. Cool, add 30 ml. of water and transfer to a separatory funnel. Rinse the flask with 100 ml. of diethyl ether, add to the separatory funnel, and shake. Separate the water layer and extract the water with 60 ml. of diethyl ether. Wash the combined ether extracts with 100-ml. portions of water until alkali-free, as tested with phenolphthalein, and dry with anhydrous sodium sulfate. Dilute with diethyl ether to 200 ml. and evaporate an aliquot of the ether extract under vacuum while bubbling carbon dioxide through. Take up in chloroform and read carotene directly. Another aliquot of the sample is appropriate for estimation of vitamin A.

Blood plasma.[58] Add 3 ml. of 95% ethanol to 3 ml. of sample. Extract the carotene with three 5-ml. portions of petroleum ether. Evaporate the combined petroleum ether extracts and take up the residue with chloroform. Read directly and use the same solution for determination of vitamin A with antimony chloride-glycol dichlorohydrin.

Procedure—The wave length for direct reading varies somewhat according to the solvent. Read in isopropanol at 450 mμ,[59] in butanol saturated with water at 422-32 mμ,[60] in petroleum ether at 450 mμ[61] or 460 mμ,[62] in petroleum ether containing 4% of acetone at 436 mμ.[63]

By perchloric acid.[64] Dilute a 0.1-ml. aliquot containing 0.003-0.03 mg. of carotene in chloroform with 1.6 ml. of amyl acetate. Add 0.4 ml. of concentrated hydrochloric acid, shake, and let stand at room temperature for 4-5 minutes. Add 2 ml. of 1:1 perchloric acid. Read the bluish-green color at 750 mμ against a reagent blank containing 0.1 ml. of chloroform.

Carotene and vitamin A simultaneously. Follow the procedure for carotene alone using an aliquot containing 0.003-0.03 mg. of carotene and

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[57] R. Sasaki, T. Tsugo, and S. Koyama, *Intern. Dairy Congr. 13th Congr. Hague* 4, 645-7 (1953).

[58] Tetsuzo Takahashi, *Vitamins (Japan)* 5, 350-3 (1952); Cf. O. A. Roels and M. Trout, *Am. J. Clin. Nutrition* 7, 197-202 (1959).

[59] R. Sasaki, T. Tsugo, and S. Koyoma, *Intern. Dairy Congr. 13th Congr. Hague* 4, 645-7 (1953).

[60] Manlio Brogioni, *Chimica (Milan)* 33, 235-43 (1957).

[61] Stig Kyrning, *Intern. Z. Vitaminforsch.* 24, 263-71 (1952).

[62] Tetsuzo Takahashi, *Vitamins (Japan)* 5, 350-3 (1952).

[63] H. L. Mitchell and H. H. King, *Anal. Chem.* 20, 637-8 (1948).

[64] Peter Flesch, *Proc. Soc. Exptl. Biol. Med.* 84, 148-9 (1953).

0.003-0.03 mg. of vitamin A, ending with "add 2 ml. of 1:1 perchloric acid." Add 0.3-0.4 ml. of absolute ethanol and shake vigorously until the layers are miscible. Read at 525 mμ and 750 mμ from 5 to 15 minutes after the addition of perchloric acid. Calculate the carotene and vitamin A concentrations by making a direct estimate of the carotene present from the reading at 750 mμ, since the absorbancy of vitamin A is negligible at this wave length. Determine the amount of carotene contributing to the reading at 525 mμ and calculate the vitamin A concentration from the remaining absorbancy at 525 mμ.

CHAPTER 3

ALCOHOLS AND THEIR ESTERS

The reaction for conversion of propylene glycol to acetaldehyde by periodic acid, page 44, is also applicable to 2-methyl-1-propanol, 2-methyl-2-propanol, and cyclohexanol. Adonitol is determined by its color in 79% sulfuric acid, page 168. Mannitol is determined as formaldehyde after oxidation with periodate, a method also applicable to glycerol. The technic as applied to sorbitol appears on page 53. Mannitol is also determined by its color in 79% sulfuric acid, page 168. The color developed with *p*-hydroxybenzaldehyde, page 45, is applicable to determination of 2,3-butylene glycol, pentamethylene glycol, 2,4-amylene glycol, and hexamethylene glycol. Butanol and ethyl Cellosolve are determined simultaneously by Sudan III, page 40. Lignins are determined by alcoholic reactions as well as phenolic, page 135.

Alcohols in General

The reaction with ceric ammonium nitrate (Vol. III, pp. 39-40) has been applied specifically to methanol, ethanol, and isopropanol below 0.1% in aqueous solution.[1]

The reaction of nitric acid on alcohols to give alkyl nitrites gives a wide absorption band in the range of 320-400 mμ with peaks at 380, 355, and 345 mμ.[2] The nature of the alkyl radical has only a negligible effect on the absorption which is best read at 360 mμ. Individual alcohols are determined with a relative error of 1-20% and the sums of alcohols can be determined with a calibration curve prepared with butanol with a relative error of 11-21%, methanol excepted. The alcoholic hydroxyl group content of aliphatic and alicyclic alcohols is determined after esterefication with 2,4-dinitrohydrocinnamic chloride (2,4-dinitro-β-phenyl propionic chloride).[3]

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[1] V. W. Reid and D. G. Salmon, *Analyst* **80**, 704-5 (1955); V. Kratochvil and C. Soběslavský, *Chem. průmysl.* **6**, 515-7 (1956).

[2] S. A. Shchukarev, S. N. Andreev, and I. A. Ostrovskaya, *Zhur. Anal. Khim.* **9**, 354-8 (1954).

[3] J. P. Riley, *J. Chem. Soc.* **1952**, 2108-10.

In the presence of glacial acetic acid sulfuric acid acts on alcohols to produce unsaturated hydrocarbons. These then react at the double bond.[4] The reagent is 4% *p*-dimethylaminobenzaldehyde in 50% sulfuric acid. With 3 ml. of glacial acetic acid, 3 ml. of concentrated sulfuric acid, and 0.5 ml. of reagent the reaction with 0.01-0.8 mg. of sample occurs at 100° in 1 hour with all alcohols. Hydroxy acids and unsaturated compounds also react. When the temperature is 60° for 1 hour the reaction occurs with unsaturated compounds, secondary and tertiary alcohols, and with iso-alcohols branched in the β position. At 19-21° for 15-30 minutes only tertiary alcohols and cholesterol react. For lower alcohols the reaction mixture must be diluted at the end of the reaction, usually to half-concentration. At 19-21° with 1 ml. of glacial acetic acid, 5 ml. of concentrated sulfuric acid, and 0.5 ml. of reagent the unsaturated primary alcohols react. The reaction does not occur with propanol and butanol when admixed with alcohols over C_5.

Sample—*Hydrocarbons.* Extract methanol, ethanol, or isopropanol from a 500-ml. sample with 20 ml. of water by mechanical stirring. Determine alcohols in the aqueous layer by ceric ammonium nitrate.

Procedure—*By ceric ammonium nitrate.* To 10 ml. of sample containing up to 0.1% of methanol, ethanol, or isopropanol, add 4 ml. of clear 66.7% ceric ammonium nitrate reagent (Vol. III, pp. 39-40) and mix for 5 minutes. Read at 486 mμ against a water blank similarly prepared. If only a single alcohol is present translate to weight per cent.

As alkyl nitrites. To a 20-ml. sample, add 20 ml. of sulfuric acid-washed petroleum ether, 1 ml. of 1:1.5 hydrochloric acid, and 2 ml. of 25% sodium nitrite solution. Shake for 5 minutes and transfer the petroleum ether layer to 20 ml. of 10% sodium bicarbonate solution or 0.4% sodium hydroxide solution. Shake to remove oxides of nitrogen and read the petroleum ether layer against a petroleum ether blank at 360 mμ.

By 2,4-dinitrohydrocinnamic chloride. As reagent nitrate hydrocinnamic acid with 3:1 concentrated nitric-sulfuric acid. Crystallize the resulting 2,4-dinitrohydrocinnamic acid from water and react with sulfuroxychloride. Esterify the sample by heating at 90° for 1 minute with excess of the reagent. Isolate the reaction product and read at 242 mμ.

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[4] W. Weigel, *Pharmazie* **12**, 357-63 (1957).

METHANOL AND METHOXYL GROUPS

Methanol oxidized by potassium permanganate yields formaldehyde which is determnied photometrically by chromotropic acid, 4,5-dihydroxy-2,7-naphthalenedisulfonic acid (Vol. III, pp. 45-6),[5] or fuchsin-sulfurous acid (Vol. III, p. 45).[6] The absorbance, using chromotropic acid as the reagent, is directly proportional to the quantity of formaldehyde which in turn is proportional to the methoxyl and methanol. The color intensity is also a function of temperature and therefore the standards and samples must be read at the same temperature.

Since the oxidation of methanol by acid permanganate is a reaction in which less than a 50% yield of formaldehyde is present under equilibrium conditions, this step is one of the most critical in the procedure. The addition of ethanol to the distillate does not improve accuracy.[7] If the oxidation takes place in the absence of ethanol, both the sample and permanganate must be at ice bath temperature to prevent the oxidation of methanol to formic acid or carbon dioxide.[8] For determination in body fluids the methanol is steam-distilled before oxidation.[9] While esters and acetal are readily hydrolyzed by distillation from 10% sulfuric acid, a few compounds require fuming with concentrated sulfuric acid.

Interference from acetaldehyde formed by the oxidation of any ethanol present is minimized by using a reference blank containing the same amount of ethanol as the sample. Compounds containing methylene dioxyl or other labile methylene groups interfere since they can be converted to formaldehyde.[10] Over 15 mg of fusel oil per ml. interferes. Glycollic acid and methyl iodide interfere. Alkoxy and alkimide groups do not interfere. The sensitivity of this method with chromotropic acid is 10-20 times as great as that of the fuschin-sulfurous acid method.[11] With the latter, color development is a function of time and temperature with the maximum in 3 hours at 28° and in 6-7 hours at 15°.[12] For determination with chromotropic acid in ethanol diluted below 6% there is almost no interference by acetic acid, acetaldehyde, or diacetyl. The ethanol must not contain more than 0.16% of fusel oil.[13]

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[5] W. Deckenbrock and M. Sprick, *Branntweinwirtschaft* **75**, 1-4 (1953).

[6] O. T. Khachidze, *Sadovodstvo, Vinogradarstvo i Vinodelie Moldavii* **12**, No. 1, 32-3 (1957).

[7] M. Langejan, *Pharm. Weekblad* **92**, 667-70 (1957).

[8] A. P. Mathers and M. J. Pro, *Anal. Chem.* **27**, 1662-4 (1955).

[9] Frank Stratton, *Tech. Bull. Registry Med. Technologists* **25**, 179-83 (1955); *Am. J. Clin. Pathol.* **25**, 1071-5 (1955).

[10] M. Beroza, *Anal. Chem.* **26**, 1970-3 (1954).

[11] S. Yamamura and T. Matsuoka, *J. Soc. Brewing, Japan* **49**, 111-5 (1954).

[12] Reiichi Hayashibe, *Ibid.* **50**, 532-4 (1955).

As little as 0.2% of methanol in organic solvents can be determined as an azo dye by conversion to gaseous methyl nitrite, reaction with 1,4-aniline sulfonic acid, and then with 1-naphthylamine.[14] As a modification of the classical Zeisel method for methoxyl the methyl iodide is absorbed in pyridine. The resulting methyl iodide-pyridinium salt is read at 366 mμ.[15]

Sample—*Methoxyl group.*[16] In a flask attached to a reflux condenser, place a 0.1-gram sample and add approximately 10 ml. of concentrated sulfuric acid through the condenser. Heat to fumes of sulfur trioxide and fume for 5 minutes to hydrolytically cleave the methoxyl group to methanol. Cool and dilute with 75 ml. of water added through the condenser. Cool and remove the condenser. Attach a distilling head and distil about 45 ml. into a flask containing 3 ml. of 95% ethanol. Dilute to 50 ml. with water.

Place a 1-ml. aliquot in an ice bath and add 2 ml. of chilled 3% potassium permanganate solution in 3:17 phosphoric acid. Let stand for 30 minutes to complete oxidation and then destroy the excess oxidant with 0.2-0.3 gram of sodium bisulfite. Develop with chromotropic acid.

Microdetermination. In a flask attached to a reflux condenser, heat a 1-mg. sample with 10 ml. of concentrated sulfuric acid to fumes for 5 minutes. Cool and add 40 ml. of water through the condenser. Remove the condenser, attach a distilling column, and reflux for 20 minutes. Maintaining a reflux ratio of 30:1, collect 2 ml. of distillate. To 1 ml. of distillate add 1 drop of 95% ethanol, cool in an ice bath, and add 2 ml. of chilled 3% potassium permaganate solution in 3:17 phosphoric acid. Allow the oxidation to proceed for 30 minutes. Destroy excess oxidant with solid sodium bisulfite. Develop with chromotropic acid.

Methanol in solutions containing ethanol.[17] Dilute the sample to an ethanol concentration of 5-6% and distil a 50-ml. portion, collecting 40 ml. of distillate. Dilute to 50 ml. with water. Add 1 ml. of distillate containing less than 0.05% of methanol to 2 ml. of chilled potassium permanganate solution containing 3 grams of potassium permanganate and 15 ml. of 85% phosphoric acid per 100 ml. of water. Place in an ice bath for 30 minutes. Decolorize with solid sodium bisulfite and develop with chromotropic acid.

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[13] A. Kamibayashi, M. Miki, and H. Ono, *Hakkô Kyôkaishi* **16**, 165-73 (1958).

[14] Y. Takayama, C. Kurata, and K. Enomoto, *Bunseki Kagaku* **5**, 449-52 (1956).

[15] H. Sobue, A. Matano, and T. Arai, *Sen-i Gakkaishi* **15**, 21-3 (1959).

[16] A. P. Mathers and M. J. Pro, *Anal. Chem.* **27**, 1662-4 (1955).

[17] Alex P. Mathers, *J. Assoc. Offic. Agr. Chemists* **38**, 753-6 (1955)

In solutions containing large amounts of ethanol.[18] To a 1-ml. aliquot of sample containing up to 0.02 mg. of methanol, add 0.2 ml. of 5% potassium permanganate in 1:17 phosphoric acid and let stand for 20 minutes at 25-30°. Shake with 0.2 ml. of 20% sodium sulfite solution to remove excess permanganate. Develop with chromotropic acid.

Cordials and liqueurs.[19] Dilute a sample containing 20-25 ml. of absolute methanol to 100 ml. with water, distil, and collect 50 ml. of the distillate. To the distillate add 4 grams of sodium chloride for each 10 ml. of water and let stand for several hours to complete the saturation. Wash out with 10 ml. of a saturated sodium chloride solution and extract with 25 ml. of petroleum ether. Shake the aqueous layer with 25 ml. of petroleum ether, separate, and re-extract the aqueous layer with 25 ml. of petroleum ether. Wash the separatory funnels used with 25 ml. of a saturated sodium chloride solution and add the washings to the combined aqueous layers. Repeat the washings with another 25-ml. portion of saturated sodium chloride solution. Distil and collect 48-49 ml. Adjust to a total alcoholic content of 5-6% and develop with chromotropic acid.

Lemon, orange, and lime extracts and flavors.[20] Dilute a 50-ml. sample to 200 ml. with water and let stand or centrifuge to separate the oil into a clear layer on top. Shake well with 5 grams of magnesium carbonate and filter quickly. Dilute 100 ml. with 50 ml. of water and distil almost 100 ml. Dilute to 100 ml. with water, mix, and develop with chromotropic acid.

Procedure—*By chromotropic acid.*[21] Add 1 ml. of 4% chromotropic acid solution to the methanol aliquot oxidized to formaldehyde and slowly add 15 ml. of concentrated sulfuric acid with swirling. Heat at 55-60° for 30 minutes and dilute with water. Cool to room temperature and dilute to 50 ml. with water. Read at 570 mμ against a reference blank.

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[18] S. Yamamura and T. Matsuoka, *J. Soc. Brewing, Japan* **49**, 111-5 (1954).

[19] "Official Methods of Analysis of the Association of Official Agricultural Chemists," 8th ed., p. 144. Association of Official Agricultural Chemists, Washington, D. C (1955).

[20] *Ibid.*, p. 325.

[21] A. P. Mathers and M. J. Pro, *Anal. Chem.* **27**, 1662-4 (1955).

Prepare the reference blank and standard methanol color by treating respectively, 1 ml. of 5.5-6% ethanol and 1 ml. of methanol containing up to 20 mg. per 100 ml. of 5.5% ethanol in the same manner as the sample, beginning with the oxidation step.

Calculate methoxyl as follows:

$(A_s/A_m) \times F \times M \times R =$ wt. % of methoxyl
A_s is absorbance of sample
A_m is absorbance of methanol standard
F is dilution factor of sample
M is % by weight of methanol in standard
R is molecular weight ratio of methoxyl to methanol.

Microdetermination. To the aliquot containing methanol oxidized to formaldehyde, add 1 ml. of 4% chromotropic acid solution and 6 ml. of concentrated sulfuric acid. Heat at 55-65° for 30 minutes and cool. Dilute to 10 ml. with concentrated sulfuric acid. Read at 570 mμ against a blank containing 1 drop of 95% ethanol. Calculate the methoxy content by the preceding equation.

By fuchsin sulfurous acid.[22] To an aliquot of distilled sample, add 5 ml. of 1% potassium premanganate solution, and 0.2 ml. of concentrated sulfuric acid. After 3 minutes add 1 ml. of 8% oxalic acid solution to decolorize the permanganate. When the mixture turns yellow, add 1 ml. of concentrated sulfuric acid and 5 ml. of fuchsin sulfurous acid. Dilute to 50 ml. and take a 10-ml. aliquot for reading at 580 mμ.

By aniline sulfonic acid.[23] Convert the methanol to methyl nitrite by shaking with sodium nitrite and dilute hydrochloric acid in dioxane. Collect the gases formed and add 1,4-aniline sulfonic acid in 1:10 hydrochloric acid. Follow with 1-naphthylamine in 1:10 hydrochloric acid. After 2 minutes make the solution alkaline with 4% sodium hydroxide solution. Read at 480 mμ.

ETHANOL

The oxidation of ethanol to acetaldehyde or acetic acid (Vol. III, pp. 46-50) and the photometric determination of the oxidation product or excess reagent constitute the major methods for the estimation of ethanol. In addition to the classic potassium dichromate as the oxidizing agent, a mixture of sulfuric acid and vanadium pentoxide has been used with determination of the blue reduction product.[24] Permanganate is also used in 1:1 sulfuric acid and the excess read at 529 mμ.[25]

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[22] O. T. Khachidze, *Sadovodstvo, Vinogradarstvo i Vinodelie Moldavii* 12, No. 1, 32-3 (1957).

[23] Y. Takayama, C. Kurata, and K. Enomoto, *Bunseki Kagaku* 5, 449-52 (1956).

Ethanol is either placed in a diffusion vessel with the oxidizing agent[26] or is diffused into the oxidizing agent.[27] Excess dichromate may be read directly or determined in acid solution with diphenylcarbazide at 550 mμ.[28] Methanol, paraldehyde, formaldehyde, and acetone interfere. The presence of ketone bodies yields a dichromate reduction equivalent to not more than 0.05% of ethanol. Another version of the oxidizing agent in the center compartment of a diffusion cell is 94 ml. of concentrated nitric acid and 0.277 gram of potassium dichromate in water diluted to 250 ml. with water.[29] The outer cell contains the sample diluted with 10 volumes of half-saturated sodium carbonate solution. After 2.5 hours at 50° the center compartment is read at 366 mμ.

Ethanol oxidized to acetaldehyde is measured as the thiosemicarbazone. Methanol, formaldehyde, lactic acid, pyruvic acid, β-hydroxybutyric acid, and acetone do not interfere.[30]

Procedure—*Biological fluids. Oxidation in a diffusion vessel.*[31] Place 3 ml. of 0.4262% potassium dichromate solution in 1:1 sulfuric acid in the center well of a Conway cell. Each ml. of this solution is equivalent to 1 mg. of ethanol. Add 1 ml. of 20% sodium carbonate solution to the outer cell to speed up diffusion of the ethanol. Partially cover so that a portion of the outer compartment is open. Add 0.5 ml. of the sample containing up to 0.55 mg. of ethanol per ml. to the outer compartment through the uncovered portion and cover completely. Tilt the entire cell several times to allow liquids to cover the surface. Incubate at 90° for 20 minutes. Remove the contents of the center well, rinse the well with water twice, dilute to 25 ml., and read at 450 mμ against suitable standards. Alternatively, read the excess dichromate directly at 465 mμ[32] or at 366 mμ.[33]

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[24] Ernst Vidic, *Arzneimittel-Forsch.* **4**, 411-8 (1954).

[25] D. Lester and L. A. Greenberg, *Quart. J. Studies Alc.* **19**, 331-5 (1958).

[26] E. M. P. Widmark, *Biochem. Z.* **131**, 473-84 (1922); O. Schmidt and R. Manz, *Klin. Wochschr.* **33**, 82-5 (1955).

[27] I. Sunshine and R. Nenad, *Anal. Chem.* **25**, 653-5 (1953); R. Besson, *Rev. franç. études clin. et biol.* **1**, 1150-1 (1956); Cf. F. R. Weedon, J. H. Gustafson, and J. D. Rolfe, *N. Y. State J. Med.* **51**, 2744-5 (1951).

[28] E. Pfeil and H. J. Goldbach, *Klin. Wochschr.* **33**, 536 (1955).

[29] D. Monnier, W. F. Rüedi, and M. Fasel, *Mitt. Gebiete Lebensm. u. Hyg.* **48**, 146-51 (1957).

[30] O. Schmidt and R. Manz, *Klin. Wochschr.* **33**, 82-5 (1955).

[31] L. A. Williams, R. A. Linn, and B. Zak, *Clin. Chim. Acta* **3**, 169-72 (1958).

By semicarbizide. Deproteinize a 0.5-ml. sample with 1 ml. of 1:9 perchloric acid and centrifuge. Add to the outer chamber of a diffusion vessel, 5 ml. of an oxidizing solution containing 5 grams of potassium dichromate and 0.5 gram of chromic chloride in 100 ml. of 1:35 sulfuric acid. Add 0.2 ml. of the deproteinized sample. In the inner absorption chamber place 5 ml. of 0.0091% thiosemicarbizide solution in 1:350 sulfuric acid. Allow the oxidation to proceed for 3.5 hours at 30°. Read the acetaldehyde-thiosemicarbazone in the inner chamber at 261.5 mμ.

By brucine.[34] To the oxidized sample from the Conway cell add 3 ml. of 1% brucine solution in 1:9 sulfuric acid. Dilute to 25 ml. and after 5 minutes read at 425 mμ.

ISOPROPANOL

Isopropanol is oxidized to acetone (Vol. III, p. 50) and developed with furfural to form difurfurylidene-acetone.[35] Acetone is either absent or corrected for. The color developed in acid solution is red to violet.

Procedure—Reflux a sample containing 0.025-0.5 mg. of isopropanol with 25 ml. of a mixture of 270 ml. of water, 80 ml. of concentrated sulfuric acid, and 60 grams of potassium bichromate. After 30 minutes oxidation distil off 50 ml.

To a 5-ml. aliquot in an all-glass distilling apparatus add 5 ml. of 40% potassium cyanide solution. Distil 2.5 ml. into a tube containing 0.2 ml. of 5% furfural solution in methanol and 0.5 ml. of 10% sodium hydroxide solution. Let the distillate stand for 30 minutes and add 15 ml. of 1:2 sulfuric acid. Read at 425 mμ.

BUTANOL AND ETHYL CELLOSOLVE

Sudan III, 1-(*p*-phenylazo)-phenylazo-2-naphthol, changes from a red to violet in inorganic aqueous bases at pH 10 or greater, when it is solubilized by some water-miscible organic solvent.[36] Since ethyl Cellosolve (2-ethoxyethanol) solubilizes Sudan III in basic solutions whereas butanol does not and butanol and ethyl Cellosolve act independently in extracting the dye, these solvents can be determined simultaneously with Sudan III. The method is applicable at 0.2-1% of solvent. Interferences from primary aliphatic amines up to *n*-butylamine and dimethylamine are overcome by distilling the alcohols from a strongly acidified solution. Other solvents present in percentages comparable to ethyl Cellosolve and butanol do not interfere, nor do dissolved salts, amines, and oils.

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[32] O Grüner, *Deut. Z. ges. gerichtl. Med.* **44,** 771-2 (1956).

[33] D. Monnier and M. Fasel, *Mitt. Gebiete Lebensm. u. Hyg.* **47,** 141-8 (1956); Cf. B. Drevon, *Compt. rend. soc. biol.* **144,** 1059-60 (1950).

[34] L. A. Williams, R. A. Linn, and B. Zak, *Clin. Chim. Acta* **3,** 169-72 (1958).

[35] Henri Etienne, *Ind. chim. belge* **17,** 455-8 (1952).

Potassium carbonate is added to the Sudan III reagent to perform 3 functions. As a base it effects the color change of Sudan III when ethyl Cellosolve is present. It salts out the organic phase. It also minimizes the solubility of the base in the organic phase so that the test is less sensitive to interferences from other water-miscible solvents such as methanol.

Procedure—To prepare the Sudan III reagent, shake 0.02 gram of dye with 200 grams of dried sodium chloride as a carrier until the crystals are uniformly coated with dye. Add 500 grams of 20-40 mesh anhydrous potassium carbonate and shake until the mixture is uniform. Keep the reagent moisture-free.

Distil a 10-ml. sample with 10 ml. of 1:18 sulfuric acid and collect 10 ml., filtering if necessary to remove suspended solids. Uniformly wet 7 grams of Sudan III reagent with 2 ml. of distillate. As the distillate soaks in, note the color change and extent of extraction of the Sudan III. Stir slowly and again observe the color and extent of extraction of the dye. Compare these color changes with standards and with Table 2. If the determination yields less than 0.2%, add 2 ml. of water to the wet reagent and stir. No increase in dye extraction should occur.

TABLE 2. CRITERIA FOR ANALYSIS OF BUTANOL AND ETHYL CELLOSOLVE BY SUDAN III

1-Butanol	%	*Ethyl Cellosolve*
No extraction or color change	0.0	No extraction or color change
Little dye extracted, red, disappears on mixing	Trace-0.2	Mixture turns violet with no noticeable extraction of dye
Definite extraction of red globules stable on mixing	0.2-0.5	Violet "dots" extracted, stable on mixing
Large red droplets extracted	0.5-1.0	Large violet droplets extracted

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[36] D. H. Feldman and J. C. Cavagnol, *Anal. Chem.* 28, 1746-8 (1956).

Fusel Oil

The concentration of fusel oil, which is predominately amyl and isoamyl alcohols, is determined by treatment with an aldehyde in the presence of a high concentration of sulfuric acid (Vol. III, pp. 50-3).

Procedure—*By anisaldehyde.*[37] Add 2 ml. of 0.5% anisaldehyde in concentrated sulfuric acid to 1 ml. of sample with cooling. Heat at 100° for 5 minutes and cool at once. Add 3 ml. of water over a period of 5 minutes, with cooling, and read at 580 mμ after 20 minutes. The same maximum applies to butanol, isobutanol, and isoamyl alcohol.

By vanillin.[38] Substitute vanillin for anisaldehyde in the preceding echnic. Read at 440 mμ after 30 minutes, when the color intensity will have become constant. The method is applicable up to 0.4% of isoamyl alcohol. Color from propanols is negligible but that from isobutanol is about 1.8 times as intense as that from isoamyl alcohol.[39]

2,5-Furandimethanol, 2,5-bis(hydroxymethyl)furan

For direct reading the maximum is at 234 mμ.[40] There is interference by bis(5-formyl-2-furfuraldehyde) ether, 5-hydroxymethyl-2-furoic acid, 5-hydroxymethyl-2-furfural, and furfural.

Tetrahydro-2,5-furandimethanol, 2,5-bis(hydroxymethyl) tetrahydrofuran

This is read directly at 288 mμ.[41] There is serious interference by bis(5-formyl-2-furfuryl) ether, by 5-hydroxymethyl-2-furfural, and by urfural. Less serious interference is by 5-hydroxymethylfuroic acid and ,5-bishydroxymethylfuran.

Ethylene Glycol, Glycol, Ethanediol

A sensitive specific color reaction is obtained when ethanediol is heated with 2,3-diaminophenazine in acetic acid. The excess reagent is tetrazotized and the tetrazonium salt formed is reduced with hypophosphorous acid.[42] The color is possibly the result of the formation of pyrazino(*b*)-phenazine. Sugars, progenitors of ethanediol, 2,3-butanedione, and ascorbic acid do not interfere.

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[37] M. Tomoeda and H. Onozalki, *Nippon Nôgei-kagaku Kaishi* **31**, 612-5 (1957).

[38] S. Shichiji and Y. Takahara, *Rept. Fermentation Research Inst.* **9**, 197-200 (1953).

[39] Toshimasa Watarai, *Nippon Jôzô Kyôkai Zasshi* **51**, 554-8 (1956).

[40] J. H. Turner, P. A. Rebers, P. L. Barrick, and R. H. Cotton, *Anal. Chem.* **26**, 98-901 (1954).

[41] *Ibid.*

Procedure—To a 1-ml. aliquot containing not more than 0.15 micromole of ethanediol, add 1 ml. of 0.021% 2,3-diaminophenazine solution in 1:1 acetic acid. Heat for 10 minutes at 100°. Cool in ice. Add 1 ml. of chilled 0.20% potassium nitrite solution and 0.5 ml. of 1:1 hypophosphorous acid. Heat for 20 minutes at 100° and cool. Dilute to 4 ml. by adding 0.5 ml. of glacial acetic acid to prevent turbidity in samples containing high concentrations of 2-propanol. Read at 600 mμ.

Ethanediol and propylene glycol simultaneously. Follow the procedure for ethanediol alone and read at 600, 610, and 715 mμ. The amount of 2-oxopropanol is determined at 715 mμ and its contributions to the absorbancies at 600 and 610μ are subtracted to measure ethanediol.

ETHYLENE GLYCOL, PROPYLENE GLYCOL, DIETHYLENE GLYCOL, 2,3-BUTYLENE GLYCOL, GLYCERINE, AND POSSIBLY OTHER POLYOLS

From 0.001-0.25 mg. per ml. of the above polyols is determined by quantitative oxidation with dichromate in 1:1 sulfuric acid followed by spectrophotometric measurement of the resulting chromic ion at 610 mμ.[43] This approach is preferable to measuring excess dichromate. Dichromate is chosen as the oxidizing agent over periodate since periodate is not a satisfactory oxidant for the small quantities of reducing agent encountered and fails to oxidize nonvicinal polyols. Absence of other reducing agents is essential. The method has been successfully applied to each of the polyols cited. An alternative is oxidation of ethylene glycol to formaldehyde by periodic acid (Vol. III, pp. 55-6) and determination by Schiff's reagent.[44]

For determination of ethylene glycol, diethylene glycol, propylene glycol, and dipropylene glycol by direct reading in the presence of styrene see page 14.

Procedure—With vigorous swirling, add 25 ml. of sample containing not more than 1 millequivalent of polyol to 25 ml. of 2.98% sodium dichromate solution in concentrated sulfuric acid. Heat at 100° for 15 minutes. Cool and read at 610 mμ against a reagent blank containing 25 ml. of water.

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[42] J. M. Dechary, E. Kun, and H. C. Pitot, *Ibid.* **26**, 449-52 (1954).

[43] R. Sargent and W. Rieman III, *Anal. Chim. Acta* **14**, 381-5 (1956)

[44] R. N. Harger and R. B. Forney, *J. Forensic Sci.* **4**, 136-43 (1959).

POLYETHYLENE GLYCOLS

Higher polyethylene glycols are determined in biological materials by adding trichloroacetic acid containing barium chloride and measurement of turbidity.[45] (Cf. Vol. III, pp. 61-2.)

Procedure—*Rumen fluid, urine, saliva, plasma.* To a 1-2 ml. sample add 10 ml. of water, 1 ml. of 10% barium chloride solution, and 2 ml. of 5% zinc sulfate heptahydrate solution. Let stand and filter or centrifuge. To a 4-ml. aliquot containing 0.1-0.7 mg. of polyethylene glycol add 4 ml. of 30% trichloroacetic acid solution containing 5% barium chloride. Measure the turbidity after 5 minutes.

PROPYLENE GLYCOL, 1,2-PROPANEDIOL

After conversion to acetaldehyde by oxidation with periodic acid (Vol. III, p. 56), propylene glycol is read directly at 277 mμ[46] or is developed with *p*-hydroxydiphenyl.[47] If the acetaldehyde is not absorbed in sodium bisulfite, the readings should be taken immediately since on standing it is believed that aldol and crossed Cannizarro condensation reactions occur.

The acetaldehyde is selectively separated from formaldehyde by aeration with nitrogen at a pH controlled with a phosphate buffer. If the molar ratio of formaldehyde to acetaldehyde is over 50:1, it may be necessary to suppress the formaldehyde with glycine or alanine. A dilute distillation solution is used to prevent the formation of interfering iodine. Rhamnose, 2,3-butylene glycol, acetyl methyl carbinol, and threonine interfere but borax does not. Polypropylene glycol does not interfere since only vicinal glycols are oxidized by periodic acid and only their oxidation products distil over.

Propylene glycol dehydrates and rearranges in concentrated sulfuric acid to a mixture of allyl alcohol and the enolic form of propionaldehyde. With ninhydrin in strong sulfuric acid, this mixture forms a violet-colored complex specific for propylene glycol and its polymers in mixtures of glycols.[48] This procedure is extremely sensitive and has been adapted to the determination of as little as 1 part of propylene glycol in 1000 parts of ethylene glycol.

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[45] Stig. Ḥyden, *Kgl. Lantbruks-Högskol. Ann.* 22, 139-45 (1956).

[46] Irwin M. Baumel, *Anal. Chem.* 26, 930-1 (1954).

[47] C. G. Huggins and O. N. Miller, *J. Biol. Chem.* 221, 377-83 (1956).

Total color develops in 50 minutes at 25° and remains constant for 30 minutes. An additional 5-minute waiting period is necessary since the color intensity rises after the solution is diluted with acid. Since the complex is heat-sensitive, attempts to speed color development at higher temperatures results in loss of color intensity. While 2-amino-1-propanol gives a positive reaction with the reagents, other glycols and monohydric alcohols do not interfere. Beer's law is followed for 0.005-0.05 mg. of propylene glycol.

Small percentages of 1,2-propylene glycol can be measured in ethylene glycol by the reaction with *p*-hydroxybenzaldehyde since propylene glycol forms a strong purple dye with the reagent and ethylene glycol gives only a weak reaction.[49] The purple compound is stable only in sulfuric acid and is sensitive to water. A strong maximum occurs at 494 mμ, but 540 mμ is chosen for the procedure because the maximum at 494 mμ is a function of the water content of the reaction mixture and the absorption at 540 mμ is independent of water content.

The purple dye is only obtainable with hydroxyl compounds containing more than 2 uninterrupted carbon atoms. Therefore, diglyceraldehyde, 2,3-butylene glycol, pentamethylene glycol, 2,4-amylene glycol, hexamethylene glycol, acetone, methyl ethyl ketone, ethyl isopropyl ketone, acetaldehyde, propionaldehyde, 2-methyl-1-propanol, 2-methyl-2-propanol, cyclohexanone, and cyclohexanol give strong colors with the reagent. Ethylene glycol, diethylene glycol, methanol, ethanol, methyl cellosolve, 1-propanol, 2-propanol, 1-butanol, phenol, benzyl alcohol, picric acid, formaldehyde, and lower aliphatic acids give weak or no reactions with the reagent. This reaction is also applicable to the determination of cyclohexanol and cyclohexanone in cyclohexane solutions.

For determination by 2,3-diaminophenazine see page 42. For determination by oxidation by dichromate see page 42.

Sample—*Glycol mixtures.* Dilute a 1-5 gram sample to 100 ml. with water. Take a 1-ml. aliquot and develop propylene glycol with ninhydrin.

1,2-Propanediol phosphate. To a 5-ml. sample at pH 8-9, containing no more than 2 micromoles, add 3 ml. of 0.1 *M* veronal buffer for pH 9.6, 0.5 ml. of 15% magnesium chloride solution, and 0.5 ml. of partially purified intestinal alkaline phosphates. Incubate 3 hours at 43°, add 0.5 ml.

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[48] L. R. Jones and J. A. Riddick, *Anal. Chem.* 29, 1214-6 (1957).

[49] S. Dal Nogare and J. Mitchell, Jr., *Ibid.* 25, 1376-9 (1953).

more of phosphatase, and reincubate 3 more hours. Stop enzyme action with 0.5 gram of trichloroacetic acid. Centrifuge to remove protein and adjust the pH to 7.0-7.2. Determine propylene glycol as acetaldehyde by oxidation with periodate.

Isolation by aeration with nitrogen.[50] As the apparatus set up two test tubes as absorbers in series. The nitrogen inlet to tube 1 runs to the bottom of the tube and breaks up the stream of nitrogen by passage through a sintered glass filter. The top outlet for gas then leads to the bottom of tube 2. As a buffer for pH 7.1 mix 50 ml. of 16.66% monopotassium phosphate with 32.2 ml. of 4% sodium hydroxide solution and dilute to 200 ml.

In tube 1 place the sample containing not more than 4 ml. of 1,2-propylene glycol, 3 ml. of the buffer for pH 7.1, and 1 drop of antifoam. To tube 2, add 1 ml. of 0.5% sodium bisulfite solution and 9 ml. of water. Assemble the apparatus and aerate with nitrogen for 2 hours under pressure of 5 pounds per square inch. Determine the acetaldehyde with *p*-hydroxydiphenyl.

Procedure—*By iodate and direct reading.* Mix 300 ml. of appropriately diluted sample with 100 ml. of 4.5% periodic acid solution. Distil into 10 ml. of water immersed in ice. Have the discharge tube of the condenser below the surface of the water. Collect 40 ml., raise the discharge tube above the surface of the distillate, and allow it to come to 50 ml. Read at 277 mμ.

By p-*hydroxybenzaldehyde.* To a 3-ml. sample containing up to 0.2 mg. of propylene glycol and not more than 10 mg. of ethylene glycol add 30 ± 1 mg. of *p*-hydroxybenzaldehyde. Mix and slowly add 5 ml. of concentrated sulfuric acid. Shake until clear and heat at 100° for 30 minutes. Cool to room temperature, dilute to 25 ml. with 1:1 sulfuric acid, and read at 540 mμ within 1 hour against a reagent blank.

By ninhydrin. To an aliquot containing 0.005-0.05 mg. of propylene glycol, add 5 ml. of concentrated sulfuric acid and mix. Stopper and heat at 70° for 10 minutes. Remove and cool to 25°. Add 0.2 ml. of 3% ninhydrin in 5% sodium bisulfite solution and let stand for 1 hour at 25°. Dilute to 12.5 ml. with concentrated sulfuric acid, mix, and let stand for 5 minutes to develop full color intensity. Read at 595 mμ. The color is stable for at least 30 minutes.

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[50] C. A. Huggins and O. N. Miller, *J. Biol. Chem.* 221, 378 (1956).

By p-*hydroxydiphenyl.* To the sample add 0.05 ml. of 4% copper sulfate pentahydrate solution. Add 6 ml. of concentrated sulfuric acid from a buret, mixing constantly during the addition. The temperature rises to 70-80°. Place in boiling water for 5 minutes and cool below 20°. Add exactly 0.1 ml. of 1.5% solution of *p*-hydroxydiphenyl in 0.5% sodium hydroxide solution. Mix as quickly as possible and store at 30° for 30 minutes or more. Heat in boiling water for 90 seconds to dissolve excess reagent and cool to room temperature. Read at 560 mμ against a reagent blank.

2-ETHYL-1,3-HEXANEDIOL

For determination of this pest repellent it is dehydrated with sulfuric acid to an unsaturated compound and then reacted with an aromatic aldehyde.[51]

Procedure—Dilute the sample containing 0.005-0.1 mg. of ethyl hexanediol to 2 ml. with water. At this point the ethanol content should not exceed 5%. Chill and add 4 ml. of chilled 0.2% *p*-dimethylaminobenzaldehyde in concentrated sulfuric acid. Heat at 100°, suitably stoppered, for 60 minutes and chill. After 30 minutes at 25° read at 500 mμ.

3-*o*-TOLYLOXY-1,2-PROPANEDIOL, MEPHENESIN

Mephenesin is extracted from plasma and urine samples and from its principal metabolite, 3-(*o*-tolyloxy)lactic acid in an alkaline medium.[52] (Cf. Vol. III, pp. 59-61.) Then they are each read in the ultraviolet at the same wave length. Beer's law is followed up to 0.2 mg. per ml. The optimum range for determination is 0.02-0.1 mg. per ml.

Urine samples should be analyzed immediately to decrease the influence of background absorption. Since there is a relatively small amount of mephenesin in relation to 3-(*o*-tolyloxy)lactic acid in urine samples and since there is a high salt concentration, the samples are diluted and adjusted to pH 7.5-8.5 before extraction.

Procedure—*Plasma. Mephenesin.* Centrifuge a 20-ml. oxalated blood sample immediately after withdrawal. Avoid hemolysis which increases background absorption. Extract 10 ml. of plasma by shaking with four 10-20 ml. portions of ether for 1 minute and allow the layers to separate.

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[51] M. C. Bowman, M. Beroza, and F. Acree, Jr., *J. Agr. Food Chem.* 7, 259-61 (1959).

[52] A. R. Maass, P. L. Carey, and A. E. Heming, *Anal. Chem.* 31, 1331-4 (1959).

To separate the emulsion which may form upon shaking, centrifuge or add sodium chloride. Extract the sample a fifth time and read this extract to determine if the extraction has been complete. Concentrate the combined ether layers to 10 ml. for mephenesin concentrations over 0.01 mg. per ml. and to 5 ml. for concentrations below 0.1 mg. per ml. Read at 300 and 270 mμ against a plasma blank which has been obtained from the blood prior to the administration of the mephenesin.

3-(o-Tolyloxy)lactic acid. Adjust the plasma from which mephenesin has been extracted to pH 3 with dilute hydrochloric acid and extract with four 10-20 ml. portions of ether. Centrifuge after each extraction to break the emulsions formed by denatured and precipitated protein. If desired, saturate the sample with sodium chloride. Concentrate the combined ether extracts to 10 ml. and add to 10 ml. of 0.4% sodium hydroxide solution. Extract the plasma with another portion of ether and read at 270 mμ to insure complete extraction of 3-(*o*-tolyloxy)lactic acid from the sample. Shake the ether-sodium hydroxide mixture for 1 minute and read both layers at 300 and 270 mμ against a plasma blank. Base the calculation of 3-(*o*-tolyloxy)lactic acid on the reading of the alkaline solution. The ether should give no absorption at 270 mμ.

Urine. Mephenesin. Here some of the 3-(*o*-tolyloxy)lactic acid can be expected to be extracted with the mephenesin. Dilute a 5-ml. sample to 25 ml. with water and adjust the pH to 7.5 to 8.5. Follow the procedure as described under "Plasma," starting with "Extract 10 ml. of plasma by shaking . . ." and ending with "Extract the sample a fifth time. . . ." Concentrate the combined extracts to 5 ml. and shake with 5 ml. of 0.4% sodium hydroxide solution. Let stand overnight and read both layers at 270 mμ.

3-(o-Tolyloxy)lactic acid. Adjust the diluted sample to pH 3 and exhaustively extract with 10 ml. portions of ether. Concentrate the extracts to 10 ml. and add to 40 ml. of 0.4% sodium hydroxide solution. Follow the procedure under "Plasma" starting with "Extract the plasma with another portion of ether. . . ." Add the value for the acid extracted along with the mephenesin.

Glycerol

The excess dichromate from the oxidation of glycerol by dichromate and sulfuric acid is measured by its reaction with 1,5-diphenylcarbazide.[53]

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[53] H. D. Reese and M. B. Williams, *Ibid.* 26, 568-70 (1954).

(Cf. Vol. III, pp. 62-5.) The maximum glycerol concentration which ca be determined by any dichromate concentration is computed from th theoretical reaction. The lowest determinable glycerol concentration i that which reduces so little dichromate that the absorbancy of the violet colored complex closely approaches that of the blank. If very dilute glycerol solutions are used, blanks should be run with each new lot of sulfuri acid.

Calculated upper limits for various reagents are as follows.

Dichromate reagent no.	*Potassium dichromate solution, grams/100 ml.*	*Glycerol theoretical upper limit, mg./ml.*
1	10.0000	13.415
2	7.5000	10.061
3	5.0000	6.708
4	1.0000	1.3415
5	0.1000	0.1342
6	0.0100	0.0134

Oxidizable compounds must be absent or removed. Molybdenum, mercury ferric iron, and vanadium give some color with *s*-diphenylcarbazide at low acidities but small amounts of these metals in 0.2 *N* acid do not interfere Normal amounts of neutral salts do not interfere.

In fermentation solutions containing residual sugars and 2,3-butanediol, 2-8 ppm. of glycerol is determined as formaldehyde by chromotropic acid after oxidation with periodic acid. Excess iodate and periodate are reduced to iodide by sodium arsenite.[54] In the range of 2-8 ppm. of glycerol the precision is ±2%. For work on biological fluids the iodide is then precipitated by silver acetate, and excess silver removed with chloride ion.[55]

The blue-colored complex formed between glycerol and cupric chloride in the presence of sodium hydroxide exhibits a broad absorption band with a maximum at 630-635 mμ.[56] Beer's law is not followed, but a reproducible straight line is obtained for 17-65 mg. of glycerol. A slight excess of cupric chloride causes incomplete formation of the complex but an excess of 400-1800% as provided by the procedure yields reproducible results. Ethylene glycol and 1,2-propylene glycol interfere. Pentaerythritol, iethylene glycol, dipropylene glycol, trimethlyene gycol, and polyethylne glycol do not interfere.

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[54] M. Lambert and A. K. Neish, *Can. J. Research* 28 B, 83-9 (1950); J. Martyn Bailey, *J. Lab. Clin. Med.* 54, 158-62 (1959).

[55] D. Biesold and E. Strack, *Z. physiol. Chem.* 311, 115-20 (1958).

[56] Lawrence K. White, *Oil and Soap* 23, 323-6 (1946); Frank Spagnolo, *Anal. Chem.* 25, 1566-8 (1953).

Sample—*Glyceryl esters and resinous vehicles.*[57] Take a sample of a naximum of 10 grams to yield 0.36-1.28 grams of glycerol upon saponifiation. Dissolve in 10 ml. of benzene, warming if necessary, and add 60 nl. of 6.6% potassium hydroxide solution in 95% ethanol. Reflux for 1.5 iours. Cool to room temperature, wash down the condenser and joint with vater, and wash from the flask. Dilute to 150 ml., add 8 ml. of concenrated hydrochloric acid, and cool to room temperature. Extract with .5-ml. portions of ethyl ether until the extract is colorless. Wash the comined ether extracts with four 5-ml. portions of water, adding each washng to the aqueous phase. Discard the ether layer. Neutralize the final iqueous extract with 21% sodium hydroxide solution and heat at 100° to xpel ether. Cool to room temperature and dilute to 200 ml. Take a 10-ml. iliquot, filter if necessary, and develop as the sodium-cupri-glycerol comlex.

Table 3. Amounts of Reagents Used in Glycerol Determinations

Dichromate reagents					
No.	*ml.*	*Dilution of reaction mixture*	*Dilution No., d, Eq. 3*	*6N sulfuric acid, ml.*	*s-Diphenyl carbazide, ml.*
1	10.00	(1) Reaction mixture to 2 liters (2) 5 ml. to 500 ml. (3) 50 ml. to 100 ml.	400	3.3	1.0
2	10.00	(1) Reaction mixture to 2 liters (2) 5 ml. to 500 ml.	200	16.4	5.0
3	10.00	(1) Reaction mixture to 2 liters (2) 5 ml. to 500 ml.	200	16.4	5.0
4	10.00	(1) Reaction mixture to 2 liters (2) 5 ml. to 100 ml.	40	2.9	1.0
5	10.00	(1) Reaction mixture to 500 ml. (2) 10 ml. to 100 ml.	5	0.9	1.0
6[a]	5.00	(1) Reaction mixture to 100 ml. (2) 25 ml. to 100 ml.	0.4	0.0	1.0

[a] 5.00 ml. of glycerol solution used.

Procedure—*By 1,5-diphenylcarbazide.* Using the appropriate amount and concentration of dichromate reagent determine glycerol in a 10-ml.

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[57] Frank Spagnolo, *Ibid.,* **25,** 1566-8 (1953).

sample containing 0.01-1.30 mg. of glycerol according to the followin general procedure. For dilutions and amounts of other reagents, refer t Table 3.

Add dichromate reagent, swirl, and slowly add 20 ml. of concentrate sulfuric acid. Stopper, swirl, and heat at 100° for 5 minutes in an incline position. Cool in cold water and transfer to an appropriate size flas washing with water. Dilute to volume with water and shake. To an aliquo add sulfuric acid and *s*-diphenylcarbazide. Dilute to volume, and shak Read at 540 mμ against *s*-diphenylcarbazide in 1:150 sulfuric acid. Calculate the glycerol according to the following equation:

$$G = \frac{d\,(B - c)}{gF}$$

where G = concentration of glycerol mg. per ml.
B = concentration of chromium in diluted blank, mg. per liter
c = concentration of chromium in diluted sample, mg. per liter
d = dilution number (Table 3) computed by multiplying the tota volume in liters of the first dilution by a subsequent dilutio factor for the aliquot
F = theoretical factor 2.6356, relating the unknown glycerol concentration to milligrams of chromium reduced
g = glycerol solution used, ml.

As the sodium-cupri-glycerol complex. Neutralize the sample wit hydrochloric acid or sodium hydroxide. Dilute an aliquot containing 18 64 mg. of glycerol to 10 ml. with water. Add 10 ml. of 21% sodium hydroxide solution and 60 ml. of 95% ethanol. Slowly add 6 ml. of 10% cupric chloride solution in 95% ethanol, swirling vigorously. Stopper shake for 2 minutes, and dilute to 100 ml. with ethanol. Centrifuge a 50 ml. aliquot for 10 minutes. Read the absorbancy of the supernatant liqui at 635 mμ against a reagent blank.

SORBITOL

The oxidation of sorbitol in alkaline solution with periodate produce formaldehyde (Cf. Vol. III, p. 67). An indirect determination of sorbito is based on the measurement of this formaldehyde with chromotropi acid.[58] Interfering carbohydrates are degraded by refluxing with alkal and separated as their degradation products by ion-exchange resins whic also remove amino acids, ascorbic, lactic, and pyruvic acids. The method is readily applied to 0.001-0.01 mg. of sorbitol. The ratio of carbohydrate to sorbitol should not be too high since the recovery of sorbitol is 100% when the ratio is 1:1, 95% when the ratio is 5:1 and 80% when the ratio is 20:1. The interfering monoanhydrides of sorbitol and other substances which react with periodate are removed by paper chromatography.

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[58] L. H. Adcock, *Analyst* **82**, 427-34 (1957); J. Martyn Bailey, *J. Lab. Clin. Me* **54**, 158-62 (1959).

Samples—*Diabetic preserve.* Disperse a 200-gram sample in warm water, cool, and dilute to 1 liter. Dilute 20 ml. to 500 ml. and filter. Reflux with 0.6 ml. of 10% sodium carbonate solution and 10 ml. of water for 4 hours. Transfer to the ion-exchange column and wash through with 400 ml. of water. Dilute to 500 ml. and take a 15-ml. aliquot.

Sorbitol is also determined by the consumption of periodic acid, page 174.

Sugarless fruit squash. Dilute a 10-ml. sample to 100 ml. Reflux 1 ml. with 0.6 ml. of 10% sodium carbonate solution and 10 ml. of water for 4 hours. Transfer to the ion-exchange column and wash through with 400 ml. of water. Dilute to 500 ml. and take a 10-ml. aliquot.

Blood. As deproteinizing agents prepare 5% solutions of zinc sulfate heptahydrate and barium hydroxide adjusting the latter as necessary so that equal volumes react quantitatively.

Mix a 0.2-ml. sample in 3 ml. of water and add 0.4 ml. each of the 5% zinc sulfate solution and the 5% barium hydroxide solution. Shake and centrifuge. Reflux a 1.6-ml. aliquot with 0.6 ml. of 10% sodium carbonate solution and 10 ml. of water for 4 hours. Wash through the ion-exchange column with 200 ml. of water. Evaporate the effluent by boiling, completing the evaporation at 100°. Correct the results for a sample of sorbitol-free blood. Correct the standards since a small amount of sorbitol is lost in the protein precipitation.

Urine. Dilute a 1-ml. sample with 5 ml. of water and add 2 ml. each of the 5% zinc sulfate solution and 5% barium hydroxide solution. Continue as in the procedure for blood starting with "Shake and centrifuge. . . ."

Feces. Gradually stir 125 ml. of water into a 10-gram homogenized sample to obtain a uniform suspension. Dilute 25 ml. to 100 ml. and shake a small portion with an equal volume of benzene to extract the fats. Mix 2 ml. of the aqueous layer with 4 ml. of water and add 2 ml. each of the

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5% zinc sulfate solution and the 5% barium hydroxide solution. Continu
as in the procedure for blood starting with "Shake and centrifuge. . .

Separation of carbohydrates by ion-exchange resins. Reflux an aliquo
with dilute alkali to convert the carbohydrates to their acids. Prepare a

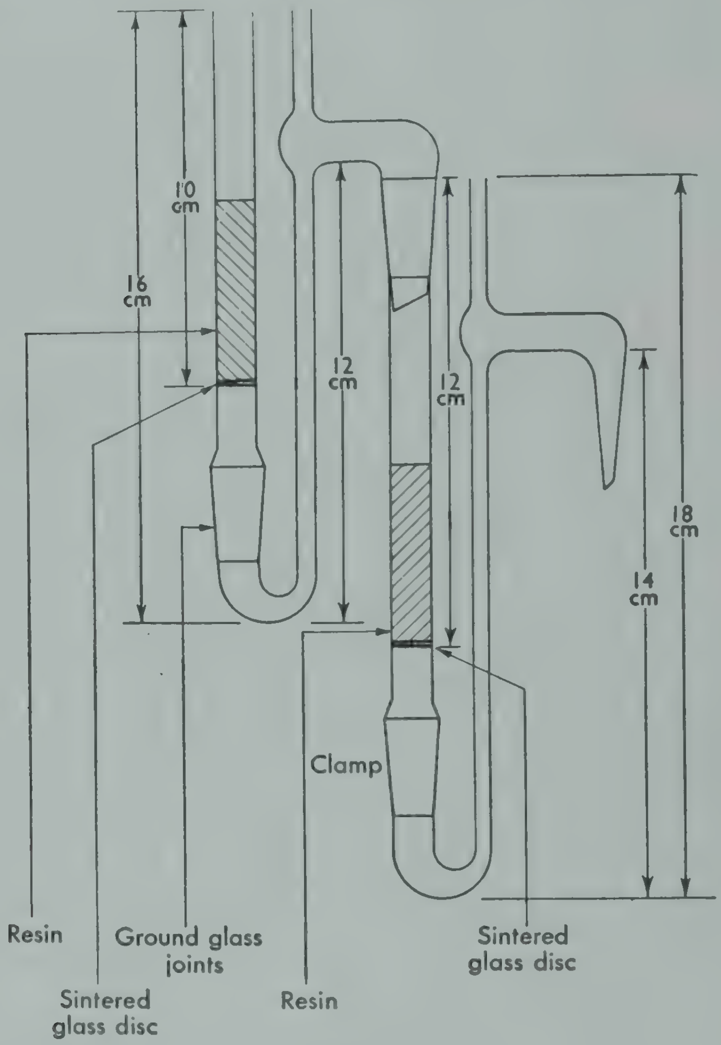
10 cm
16 cm
12 cm
12 cm
18 cm
14 cm
Clamp
Resin
Ground glass joints
Sintered glass disc
Resin
Sintered glass disc

Fig. 3

Apparatus for ion-exchange of polyhydroxyl compounds

apparatus according to Figure 3. Fill each U-tube with sufficient water t
cover the sintered glass disc and suck out any air bubble from beneat
the disc. Place 2 grams of air-dried Zeo-Karb 225 resin in the left U-tub
and place 1 gram of 60-100 mesh De-Acidite FF resin in the other tub

Wash the column of Zeo-Karb 225 with 2% sodium hydroxide solution and the other column with 1:24 hydrochloric acid. Wash both columns with water. To regenerate the columns, pass a slight excess of 1:24 hydrochloric acid through the Zeo-Karb 225 and a slight excess of 2% sodium hydroxide through the De-Acidite FF. Wash each with water until about 20 ml. of the washings require not more than one drop of 0.01 *N* acid or alkali for neutralization, using methyl red as their indicator. Join the U-tubes and wash a small volume of sorbitol through the Zeo-Karb 225 column with not less than 200 ml. of water delivered steadily to the columns over a period of several hours. Dilute the effluent or evaporate to dryness depending on the amount of sorbitol present and according to the amount required by the development method chosen.

Separation from monoanhydrides. Technical sorbitol syrup. Dilute the syrup to contain about 1 gram of total solids per 100 ml. Place equal volumes of the sample and standard solutions containing 0.25-1 gram per 100 ml. on paper which has been cut and marked for descending solvent paper chromatography. Flank the spots with marker spots of one of the standard solutions. Condition the paper for 1 hour over *n*-butanol saturated with water and develop the chromatogram with that solvent for 16 hours. Allow the solvent to evaporate completely from the paper and cut off the marker spot strips. Spray these strips with saturated potassium periodate solution and after 5-10 minutes spray with a hydrochloric acid-benzidine solution prepared by mixing 1 volume of 1:399 hydrochloric acid with 1 volume of 3.68% benzidine in 100 ml. of 90% ethanol and 50 ml. of acetone. The sorbitol and other substances which reacted with the periodate will show white or yellow against a blue background. From the unsprayed paper cut areas of similar shape and size corresponding to the sorbitol spots, standard and unknown. Elute the sorbitol from these papers by suspending them at the end of condensers over flasks or tubes holding small equal volumes of 1:3 ethanol. Reflux the ethanol solutions for 1 hour. Determine the sorbitol in the extract using the procedure described for 0.001-0.01 mg. of sorbitol.

Procedure—To prepare the chromatographic reagent, dissolve 0.5 gram of the sodium salt of chromotropic acid in 50 ml. of water, add 0.05 gram of stannous chloride dihydrate, and dilute to 250 ml. with 2:1 sulfuric acid. All operations with chromotropic acid must be shielded from bright light. Evaporate the solution containing 0.01-0.05 mg. of sorbitol to dryness at 100°. Wet with 1 ml. of a 1:1 mixture of 0.4% sodium metaperiodate solution in 1:799 sulfuric acid and a saturated sodium bicarbonate solution as the buffer. Cover and leave in the dark for at least 1 hour. Precipitate the bicarbonate and excess sodium metaperiodate by adding 1 ml. of 20% lead dithionate solution. Mix and centrifuge. To 1 ml. of the supernatant liquid, add 9 ml. of the chromotropic acid reagent. Centrifuge to separate the lead sulfate and develop the formaldehyde-chromotropic acid color in the supernatant liquid by heating it at 100° for 30 minutes. Read at 570 mμ against 2:1 sulfuric acid. For the determination of 0.001-0.01 mg. of sorbitol, carry out the above procedure, using 0.1 of the volume of the solutions described.

INOSITOL

Inositol oxidized with bromine in a neutral solution yields an intermediate product which gives a blue reduction product with phosphomolybdotungstic acid.[59] (Cf. Vol. III, pp. 67-8.) Sorbose, levulose, glucose, mannose, galactose, maltose, and lactose give 5-10% as much color as is shown by an equal amount of inositol. Sorbose, levulose, and glucose can be destroyed by heating 10 ml. of the 1% sugar solution for 30 minutes at 100° with 2 grams of recently calcined magnesium oxide. Inositol is also determined by the color in 79% sulfuric acid, page 168.

Procedure—As a reagent dissolve 100 grams of sodium tungstate and 25 grams of sodium molybdate in 700 ml. of water. Add 50 ml. of 85% phosphoric acid and 100 ml. of concentrated hydrochloric acid. Reflux for 8 hours and dilute to 1 liter. To 1 ml. of sample containing 0.1-1 mg. of inositol, add 1 ml. of bromine solution made from 1 drop of bromine and 2 ml. of water. Heat at 100° for 30 minutes, cool quickly, and treat with 1 ml. of the reagent diluted 1:10. After 5 minutes, add 1 ml. of 10% sodium carbonate solution and let stand for 1 hour. Dilute to 10 ml. and read at 560-650 mμ.

CYCLOHEXANOL

Cyclohexanol and cyclohexanone are determined in cyclohexane by *p*-hydroxybenzaldehyde by a method described under propylene glycol, pages 44-45.[60] Since cyclohexanol and cyclohexanone show a maximum at 535 mμ but at 625 mμ the absorption by cyclohexanone is negligible, the amounts of these components are calculated by simultaneous equations.

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[59] P. Balatre and M. Traisnel, *Bull. soc. pharm. Lille* 1955, No. 1, 28-9.

[60] S. Dal Nogare and J. Mitchell, Jr., *Anal. Chem.* 25, 1376-9 (1953).

Cyclohexanol heated in sulfuric acid with diphenylamine to over 100° develops a red color.[61] The maximum absorbancy is at 530 mμ. It is sensitive to 0.005 mg. per ml.

VINYL ACETATE

The vinyl acetate group in polyvinyl acetate is determined as ferric hydroxamine acid formed with hydroxylamine hydrochloride and ferric chloride similar to the determination of aryl groups.[62] Down to 0.1% of vinyl acetate monomer in the polymer the method is accurate to ±2%.

Procedure—Disperse a 10-50 mg. sample of polyvinyl acetate in 0.5 ml. of toluene. Add 4 ml. of petroleum ether and mix well. Remove the supernatant liquid and wash the remaining polymer twice with 0.5-ml. portions of petroleum ether. To the combined petroleum ether extracts add 1 ml. of 2.5% sodium hydroxide solution in 90% ethanol and 1 ml. of 2.5% hydroxylamine hydrochloride solution in 90% ethanol. Let stand at 20° for 10 minutes and then at 55° for 16 minutes. Add 10 ml. of 1% ferric chloride solution in 90% ethanol, dilute to 25 ml. with methanol, and read at 530 mμ.

POLYETHYLENE GLYCOL MONOOLEATE

The chloroform solution of the blue polyethylene glycol monooleate-cobaltothiocyanate complex has a maximum absorption at 620 mμ plus an absorption peak in the ultraviolet at 318.5 mμ.[63] The sensitivity in the ultraviolet is about 6 times as great as at 620 mμ. Beer's law is followed at 620 mμ up to 1 gram per liter and at 318.5 mμ up to 0.6 gram per-liter. It is probably that ammonium cobaltothiocyanate behaves as a dual reagent first forming a polyoxonium compound with the ammonium cation and then acting as a precipitant by means of the cobaltothiocyanate ion. The absorption is ascribed to the cobaltothiocyanate ion, $(Co(CNS_4)^=$. Results are complicated by the fact that the product is not a chemical individual and can vary in reaction from batch to batch and particularly from manufacturer to manufacturer.

The following factors are important in obtaining reproducible results: amount of reagent, ratio of reagent volume to test volume, volume of extracting solvent, method of removal of water from chloroform, and temperature of the solution before extraction. Water should be removed by centrifuging rather than by filtration. In the latter technic the blue complex is lost because it is surface-active.

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[61] A. S. Maslennikov, *Zhur. Anal. Khim.* **13**, 599-602 (1958).

[62] Yuji Takayama, *Japan Analyst* **4**, 299-302 (1955).

[63] E. G. Brown and T. J. Hayes, *Analyst* **80**, 755-67 (1955).

As an alternative to chloroform as the extracting solvent, *o*-dichlorobenzene may be employed. In this case the absorption maximum is shifted to 625 mμ. Temperatures over 26° cause low results, possibly because the complex decomposes. Low temperatures down to 10° have little effect on the accuracy of the method. Tartrazine and azogeranine, dyes used in rayon-softening solutions containing polyethyleneglycol monoleate, do not interfere. Cellulosic impurities, low molecular weight polyethylene glycol monoesters, and triethylene glycol monosterate do not interfere. Solutions of the glycol for calibration purposes should not be prepared by the dilution of concentrated solutions.

Procedure—As a reagent, dissolve 15 grams of cobalt nitrate hexahydrate and 100 grams of ammonium thiocyanate in water. Adjust the temperature to 20° and dilute to 500 ml. To 20 ml. of the ammonium cobaltothiocyanate reagent at 20°, add 5 ml. of the sample at 20°. Shake vigorously for exactly 1 minute and let stand for 5 minutes at 20°. Extract with 4 ml. of chloroform by shaking for 1 minute. Drain off the chloroform layer and repeat the extraction with three or four 4-ml. portions of chloroform. Rinse the stem of the funnel with chloroform and let the combined extracts and washings stand for 5 minutes at 20°. Dilute to 25 ml. with chloroform, shake, and centrifuge a portion for 1-2 minutes to remove water droplets. Read at 318.5 mμ or 620 mμ against chloroform.

NON-IONIC DETERGENTS

Phosphomolybdic acid gives a specific violet to rose-pink color with non-ionic detergents in concentrated sulfuric acid. When the presence of concentrated sulfuric acid in the instrument is undesirable or when greater dilution of the color is required with higher concentrations of detergent, a direct determination of molybdenum in the phosphomolybdate complex precipitate is preferred to a determination of the complex itself.[64] In such case, the molybdenum is determined by thiocyanate in the presence of stannous chloride (Vol. II, pp. 478-85, Vol. IIA, pp. 370-4). This procedure is the more sensitive. If an extraction is required, butyl acetate saturated with ammonium thiocyanate and stannous chloride is the preferred extracting solvent. The turbidity which appears is not removed by centrifuging and does not significantly increase the absorption. Similar amounts of salts of ethylene-diamine tetraacetic acid have no effect but similar amounts of alkyl sulfate interfere. Low results are caused by over 0.1 mg. of carboxymethylcellulose.

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[64] D. G. Stevenson, *Ibid.* 79, 504-7 (1954).

Procedure—*As the phosphomolybdate complex.* Dilute a sample containing 0.01-0.5 mg. of detergent to 5-10 ml. Add 3 drops of 1:4 hydrochloric acid, 2 drops of 10% barium chloride solution, and 2 drops of 10% phosphomolybdic acid solution. Stir and centrifuge. Decant the supernatant liquid and let the tube stand inverted for 1-2 minutes to drain. Washing is not necessary since the reagents do not cause any color. Dissolve the precipitate in concentrated sulfuric acid with stirring and warming if necessary. Dilute to 4 ml. with concentrated sulfuric acid. Read at 520 mμ after 40 minutes.

As molybdenum. Follow the procedure for determination as the phosphomolybdate complex precipitate, ending with ". . . Decant the supernatant liquid. . . ." Wash the precipitate with 5 ml. of water, centrifuge, and decant the supernatant liquid. Invert the tube and allow to drain. Dissolve the precipitate in 1-2 ml. of concentrated sulfuric acid and dilute to 6 ml. with water. Add 1 ml. of 5% ammonium thiocyanate solution and 0.5 ml. of 35% stannous chloride solution in 1:9 hydrochloric acid. Dilute to 10 ml. and centrifuge the amber solution if necessary. Read after 20 minutes at 470 mμ. Alternatively, extract with butyl acetate saturated with ammonium thiocyanate and stannous chloride.

α-Terpineol, α-Menthenol

A stable bluish-green color is produced by the reaction between α-terpineol in pine oil and a vanillin-hydrochloric acid reagent at 60°.[65]

Procedure—To a 15-ml. sample, as of water containing pine oil, add 5 ml. of ethanol and 30 ml. of a 1% solution of vanillin in concentrated hydrochloric acid. Shake for 1 minute and heat at 60° for 25 minutes. Cool to room temperature and shake. Read against distilled water, if the sample is clear, or against a mixture containing 15 ml. of the sample, 5 ml. of ethanol, and 30 ml. of concentrated hydrochloric acid if the sample is colored.

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[65] J. A. Sutton, *U. S. Bur. Mines Rept. Invest.* No. 4990 (1953).

TERPINOL HYDRATE; 1,8-*p*-MENTHANEDIOL

The reaction of terpinol hydrate with phosphomolybdic acid produces a blue color which turns to a stable greenish-blue.[66] A possible mechanism for the reaction is the formation of molybdenum blue, a reduction product of phosphomolybdic acid. There is no interference by menthol, eucalyptol, codeine sulfate, tincture of belladonna, ammonium chloride, extract of glycyrrhiza, fluid extract of ipecac, extract of senega, extract of lobelia, powdered ipecac, antimony and potassium tartrate, codeine phosphate, prunes virginiana, prunes strobus, and sanguinaria. There is interference from glycerophosphates. Beer's law is followed for 0.85-2.55 mg. per 100 ml.

Procedure—*Terpinol hydrate elixir.* Dilute a 5-ml. sample to 100 ml. with water. To 5 ml. of 5% phosphomolybdate solution, add 1.5 ml. of sample and 6 ml. of concentrated sulfuric acid in 2-ml. portions, mixing after each addition. Cool 15 minutes and dilute to 100 ml. with water. Read after 9 minutes against a reagent blank at 660 mμ.

Tablets. Grind twenty tablets to a fine powder. Weigh a suitable sample, add water, and let stand for 1 hour with occasional agitation. Filter and dilute so that the final solution contains 0.85-2.55 mg. per 100 ml. Develop as for elixir from "To 5 ml. of 5%. . . ."

PHYTOLS

The formation of 4-phenylazophenyl urethans of phytols, unsaturated alcohols from chlorophylls, can be used for their photometric determination.[67]

Procedure—Reflux 0.5-0.15 gram of crude phytols and 2.5 equivalents of 4-phenylazophenyl isocyanate in 15-20 ml. of benzene for 12-20 hours protected from absorption of moisture. Evaporate the benzene under vacuum to about 2 ml. and add 8 ml. of petroleum ether. Chromatograph on an alumina column saturated with petroleum ether. Elute with a 3:1 mixture of petroleum and ethyl ethers until the zone of orange-colored phytol urethans is quantitatively recovered. Evaporate and dry over phosphorus pentoxide under reduced pressure. Dissolve the resulting oil in benzene and read at 463 mμ against urethane as the standard.

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[66] H. Platt and A. E. James, *J. Am. Pharm. Assoc.* **44,** 666-8 (1955).

[67] O. Hromatka and L. Stentzel, *Monatsh.* **89,** 54-60 (1958).

VITAMIN A

The polythene alcohol, vitamin A, is read directly at 310, 325, and 334 mμ and a correction applied [68] (Vol. III, pp. 70-91). For this determination to be valid, the sample must first pass the colorimetric validity test with antimony trichloride as a reagent. For reading at 325 mμ a pH of 10.5 is preferred.[69] Vitamins E, B_1, B_6, niacin, and C do not interfere. Isopropanol is a suitable solvent.[70]

The determination of vitamin A by reaction with antimony trichloride in an anhydrous medium was acceptable so long as it was the only available method. The tendency for years has been for it to be superseded. Therefore the trend of the methods which follow is to apply other reagents, aside from direct reading of course. In the presence of interfering materials such as ethylene dichloride, the extinction by extrapolation[71] is low. An alternative solvent is 1,2-dichloroethane in place of chloroform in the preparation of the antimony trichloride reagent. Dissolve 30 grams of antimony trichloride in 100 ml. of 1,2-dichloroethane and filter. The intensity of the blue color formed with vitamin A is the same in both solvents. A green color is formed with vitamin A aldehyde. Also 0.1 mg. of carotene or 0.1 mg. of xanthophyll give a blue color equivalent to 17 and 22 USP units of vitamin A, respectively.[72]

Glyceroldichlorohydrin is a new reagent for development of vitamin A. The variations in the reagent itself according to the method of preparation are a serious problem. The temperature effect is greater than with antimony trichloride. At 25° the maximum coloration is developed in chloroform in 2 minutes. The sensitivity of the reagent is enhanced by addition of antimony trichloride.[73]

A blue color initially in the reaction of vitamin A with 2% acetyl chloride solution and 0.4-10% antimony trichloride solution in chloroform or isopropanol which changes to a red-violet color with a maximum absorption at 550 mμ.[74] The intensity of the red-purple color is proportional to vitamin A within the range of 10-60 international units per ml. Similar colors are produced by activated glyceroldichlorohydrin, vitamin D_2, and β-carotene.

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[68] "Official Methods of Analysis of the Association of Official Agricultural Chemists," 8th ed., pp. 812-14, Association of Official Agricultural Chemists, Washington, D. C. (1955); "U. S. Pharmacopoeia," fifteenth ed., pp. 941-2, Mack Publishing Company, Easton, Pennsylvania (1955).

[69] L. Via and M. Giannini, *Farmaco (Pavia) Ed. prat.* **11**, 229-34 (1956).

[70] Osvaldo Castrelos, *Rev. farm. (Buenos Aires)* **101**, 15-9 (1959).

[71] K. H. Wagner, G. Linden, and A. Stumpf, *Hoppe-Seyler's Z. physiol. Chem.* **296**, 193-204 (1954).

[72] Augusta B. McCord, *J. Lab. Clin. Med.* **42**, 660-2 (1953).

[73] C. Kawasaki and S. Suenaga, *J. Pharm. Soc. Japan* **69**, 460-3 (1949); A. Fuijita and M. Aoyama, *J. Biochem. (Japan)* **38**, 263-9 (1951); Masataro Aoyama, *Kitasato Arch. Exptl. Med.* **24**, 413-4 (1952); Tetsuzo Takahashi, *Vitamins (Japan)* **5**, 350-3 (1952); Senji Suenaga, *Eiyô to Shokuryô* **6**, 101-5 (1953-4).

A color which has a maximum absorption at 543 mμ develops upon the addition of 3:2 perchloric acid to a chloroform solution of vitamin A.[75] Another reagent is a standard phenol red preparation at pH 6.4. With this reagent the maximum absorption is at 575 mu.[76] Vitamin D does not interfere. Vitamin A is determined photometrically with 1,3-dichloropropan-2-ol activated by 1-2% hydrochloric acid. Vitamin A and carotene can be determined in the same sample using wave lengths of 550 and 750 mμ respectively.[77]

The maximum color is developed with 1,2-propylenechlorohydrin and 1,3-propylenechlorohydrin in 2 minutes at 60-70° for reading at 630 mμ. A somewhat inferior reagent is 1-chloropropylene glycol, reacted for 2 minutes at 25° and read at 550 mμ.[78]

Vitamin A is converted to anhydrovitamin A in anhydrous solvents in the presence of traces of mineral acids.[79] This displaces the maximum in benzene from 331 mμ to give maxima at 351, 371, and 392 mμ and increases the absorbancy. The reaction is probably about 84% complete. As applied to unsaponifiable extracts the change often permits estimation of vitamin A without further purification, the background absorption being the same in each. This should not be confused with the difference in spectrum obtained by destructive irradiation or selective sorption of vitamin A since the difference in spectrum of this method corresponds to no actual compound.

While hydrogen chloride in ethanol has been applied qualitatively[80] and quantitatively,[81] the reaction is better controlled by *p*-toluene sulfonic acid as catalyst in benzene.[82] This conversion of the alcohol form of the vitamin occurs almost instantaneously at room temperature. Thereafter the amount of dehydration product decreased with time. Neutralization with alkali stabilizes the compound for at least 1 hour. Small changes in room temperature have a negligible effect.

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[74] Guido Cavina, *Congr. intern. biochim., Résumés communs.*, 3^e *Congr. Brussels* **1955**, 142; *Ann. chim. (Rome)* **46**, 43-61 (1956); *Rend. ist. super. sanità* **20**, 913-22 (1957).

[75] F. Čůta and J. Celikovský, *Chem. Listy* **48**, 1346-50 (1954).

[76] *Ibid.*

[77] V. P. Vendt, *Vitaminy, Akad. Nauk, Ukr. S.S.R.* **1953**, 7-29.

[78] S. Erbe, *Z. anal. Chem.* **159**, 327-32 (1958).

[79] J. R. Edisbury, A. E. Gillam, I. M. Heilbron, and R. A. Morton, *Biochem. J.* **26**, 1164-73 (1932); Norris D. Embree, *J. Biol. Chem.* **128**, 187-98 (1939); P. Meunier, R. DuLou, and A. Vinet, *Compt. rend.* **216**, 907-8 (1943); *Bull. soc. chim. biol.* **25**, 371-8 (1943); E. M. Shantz, J. D. Cawley, and N. D. Embree, *J. Am. Chem. Soc.* **65**, 901-6 (1943).

[80] S. R. Ames and P. L. Harris, **Science** *120*, 391-3 (1954).

Measurement of the yellowish-green fluorescence of vitamin A is ten times more sensitive than the colorimetric methods.[83] Carotene and cryptoxanthin give about 12% and vitamin D_3 about 3.3% of the intensity of vitamin A on a weight basis. Saponification diminishes the fluorescence. Free vitamin A alcohol fluoresces about 20% less intensely than the vitamin A ester on the mole basis. There productibility compares favorably at 1-20 international units with photometric results.[84]

Destructive irradiation methods involve measuring absorption before and after irradiation. They permit determination of vitamin A in the presence of carotene. In this method vitamin A is read at 328 mμ, the sample is irradiated 6-8 times as long as found necessary to destroy 50% of the vitamin A in pure solution and read again at 328 mμ.[85] A correction must be made in the blank since the optical density at 328 mμ rises under photolysis.[86]

Saponification of oil samples aids in removing interferences. When the wave length is displaced from 327.5 mμ in a direct reading of vitamin A further saponification is necessary.[87] The saponification, usually carried out in nitrogen, can be performed in air without loss by use of hydroquinone as antioxidant.[88] Extinction values determined on unsaponifiable matter are lower than those obtained by other methods.

Thymyl *p*-phenylazobenzoate can be used as a standard reference for vitamin A determinations since it has a spectral absorption curve closely resembling that of vitamin A acetate, with a maximum at 325 mμ. To prepare, heat thymol with *p*-phenylazobenzoyl chloride in pyridine-benzene mixture. Recrystallize from 80% ethanol solution or isopropanol.[89]

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[81] J. Gillman, K. B. Norton, D. E. A. Rivett, and D. A. Sutton, *Biochem. J.* **63**, 458-60 (1956).

[82] Edgar M. Shantz, *J. Biol. Chem.* **182**, 515-24 (1950); P. Budowski and A. Bondi, *Analyst* **82**, 751-60 (1957).

[83] A. Fujita and M. Aoyama, *J. Biochem. (Tokyo)* **38**, 271-5 (1951).

[84] N. K. De, *Indian J. Med. Research* **43**, 391-5 (1955).

[85] O. A. Bessey, O. H. Laury, M. J. Brock, and J. A. Lopez, *J. Biol. Chem.* **166**, 177-88 (1946).

[86] K. Kimura and A. Fujita, *J. Vitaminol. (Osaka)* **3**, 155-64 (1957); Cf. C. Kawasaki and S. Suenaga, *Eiyô to Shokuryô* **6**, 62-3 (1953).

[87] R. V. Swann, *J. Pharm. and Pharmacol.* **4**, 886-97 (1952).

[88] H. Moor, *Milchwissenschaft* **8**, 298-300 (1953); H. Moor, *Mitt. Gebiete Lebensm. u. Hyg.* **44**, 257-64 (1952).

Part of the problem is interfering substances. Chromatography, employing various forms of sorbants, is employed to separate the interferences, or the interferences, such as carotene, are read directly and their contribution subtracted. The main variant in chromatography is the sorbant used. Defatted bone meal holds vitamin A alcohol sorbed while pigments including carotene or dye are washed through with petroleum ether.[90] With diatomaceous earth and magnesia as sorbants and hexane as the solvent, vitamin A, α-carotene, and β-carotene are separately eluted.[91] In this case the carotenes are used as tracers since the vitamin A band is colorless and appears between the α-carotene and β-carotene bands. Since the colorless vitamin A band is eluted before β-carotene, but not necessarily before α-carotene, it is necessary to elute all of the α-carotene layer and a small amount of the β-carotene layer to ensure complete removal of vitamin A.

In the presence of tocopherol a direct reading of vitamin A yields high results even after a correction is applied. Deactivated alumina separates α-tocopherols from vitamin A and permits direct reading of each.[92] Vitamin A alcohol can be separated from as much as 12 times its weight of α-tocopherol. The alumina sorbant must be deactivated because in its active form it sorbs the vitamin A and tocopherol too strongly to permit good separation.

Artificial coloring materials added to margarine which interfere with vitamin A determinations are removed by chromatography or extracted with glacial acetic acid containing 0.1% of acetaldehyde.[93]

Vitamin-A alcohol, vitamin-A acetate, and vitamin-A palmitate can be separated by chromatography on a polyethylene column.[94] Chloroform 20% and heptane 40% is the stationary phase. A suitable mobile phase is 55% aqueous isopropanol. The method permits reading directly in the eluate.

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[89] M. Vignau and M. Debodard, *Bull. soc. chim. biol.* **34,** 831-4 (1952).

[90] J. W. Lord and P. M. Bradley, *Analyst* **80,** 429-39 (1955).

[91] "Official Methods of Analysis of the Association of Official Agricultural Chemists," 8th ed., pp. 814-16, Association of Official Agricultural Chemists, Washington, D. C. (1955).

[92] D. T. Ewing, L. H. Sharpe, and O. D. Bird, *Anal. Chem.* **25,** 599-604 (1953).

[93] K. Naito and T. Mori, *Vitamins (Japan)* **8,** 17-20 (1955).

[94] O. Wiss and U. Gloor, *Z. physiol. Chem.* **310,** 260-4 (1958).

Separation by Chromatography[95]—*By diatomaceous earth and magnesia.* As a chromatograph tube, seal a 23 × 200 mm. tube to a 5 × 80 mm. one. Place a small cotton plug at the base. As sorbant, pass a 1:1 mixture of diatomaceous earth and magnesia through a sieve and roll on paper at least 50 times. Tightly pack 10 cm. of sorbant into the column, applying suction during packing. Add a 1-cm. layer of anhydrous sodium sulfate on top. Add a hexane solution containing 0.1 mg. of β-carotene to an aliquot of the sample as a tracer prior to passing the solution through the chromatography column, unless alfalfa meal is present.

Draw a 50-ml. aliquot of the extract containing vitamin A, β-carotene, and α-carotene through the dry column with suction. Elute with 35-40 ml. of 10% acetone solution in hexane, stopping when a small amount of the heavy β-carotene band passes through the column. The eluate should be yellow. Dilute to 50 ml. and mix. Using mild heat and reduced pressure, evaporate a suitable aliquot of the eluate and dissolve in chloroform, so that a 1-ml. aliquot will have an absorbance, after addition of antimony trichloride reagent, of approximately 0.5-0.2. Develop with the antimony trichloride reagent.

By bone meal.[96] Plug the tapered end of a tube 30 cm. in length by 1 cm. in diameter with a wad of cotton wool. Add bone meal which passed through a No. 80 sieve and is retained on a No. 170 sieve either dry or suspended in petroleum ether boiling in the range of 80-100°. When the height of the column is 22-25 cm., draw petroleum ether through the column by means of suction until all air pockets are removed leaving some petroleum ether at the top of the bone meal. Sheath the column in black paper to prevent decomposition of vitamin A. Connect the column by a side arm above the bone meal to a source of compressed air.

Prepare the unsaponifiable fraction from a 10-gram sample. Transfer quantitatively to the column using 80-100° petroleum ether. Apply air pressure so that the rate of flow is about 2 drops per second. About 15 cm. of mercury is usually sufficient pressure. Make sure some solvent remains above the bone meal. Wash the column with 50 ml. of petroleum ether until no color from carotene or dye appears in the eluate. Elute the column with petroleum ether containing 16% of diethyl ether. If the solvent is cloudy, shake with anhydrous sodium sulfate to remove water.

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[95] "Official Methods of Analysis of the Association of Official Agricultural Chemists," 8th ed., pp. 814-16, Association of Official Agricultural Chemists, Washington, D. C. (1955).

[96] J. W. Lord and P. M. Bradley, *Analyst* 80, 429-39 (1955).

Collect the eluate in 5 ml. portions in tubes. A second group of pigments appears and serves as a marker for the vitamin A which appears 3-4 tubes later. Identify the tubes containing vitamin A by testing 0.4-ml. aliquots for a blue color with anhydrous antimony trichloride reagent. Combine the aliquots containing vitamin A and dilute to 20-25 ml. with petroleum ether.

Read vitamin A directly. To regenerate the bone meal, wash with 75 ml. of petroleum ether containing 16% of diethyl ether, draw dry, and fill with petroleum ether. The sorbant can be used about 3 times by this method. Then, remove it from the tube and thoroughly wash by decantation with a 3:1:1 mixture of petroleum ether, diethyl ether, and acetone. Dry and rescreen.

By deactivated alumina.[97] *Vitamin A and α-tocopherol simultaneously.* In this separation the vitamin A esters have been saponified to the alcohol. To deactivate the alumina, spread 10 grams in thin layers in Petri dishes. Place in desiccators over 300 ml. of 3:2 sulfuric acid and let stand for approximately 48 hours to allow the alumina to come to equilibrium with the water vapor. The chromatography column should consist of three continuous parts, a top column approximately 3 cm. in diameter and 15 cm. long which serves as the solvent reservoir, a middle section of 7-mm. diameter and 12 cm. long as the column proper containing the sorbant resting on a cotton plug, and finally a narrow short section serving as the column outlet.

Fill the column with hexane to about 0.25 inch above the cotton plug. Drain and refill with hexane, lightly tapping to remove air from the cotton plug. Add 2.5 grams of sorbant and wash down with hexane. Settle by tapping the column. Apply suction so the hexane reaches 1 cm. above the surface of the alumina. Add an aliquot of the sample and wash down with three 5-ml. portions of hexane, never allowing the liquid to go below the surface of the sorbant.

Develop with a 1:2 mixture of ether and hexane. Do not stir the alumina at the top of the column. Collect the vitamin A and dilute to 25 ml. with 1:2 ether-hexane. Read at 325 mμ against the same solvent. Elute the tocopherol with 99% isopropanol and read at 290 mμ against that solvent.

Separation of free and esterified vitamin A.[98] Dissolve a 10-ml. sample, containing less than 0.01 mg. of free vitamin A and 0.02 mg. of esterified vitamin A, in 5 ml. of petroleum ether and pour through a 20 × 7 mm. column containing alumina deactivated by heat-drying and moistening successively. Elute vitamin A ester with two 2-ml. portions of petroleum ether and remove free vitamin A with two 2-ml. portions of a 1:4 mixture of acetone and petroleum ether. Determine vitamin A in both eluates with glycerol dichlorohydrin.

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[97] D. T. Ewing, L. H. Sharpe, and O. D. Bird, *Anal. Chem.* **25**, 599-604 (1953).

[98] A. Fujita and M. Aoyama, *J. Biochem.* (*Japan*) **40**, 151-6 (1953).

Samples—*General.*[99] Dilute the sample with chloroform so that an aliquot contains up to 100 USP units of vitamin A. Heat at 100° and add 0.35 ml. of 50% potassium hydroxide solution in 95% ethanol and 5 ml. of 95% ethanol. Mix and reflux, using an air condenser, for 15 minutes. Cool, dilute with 5 ml. of water, and add 10 ml. of diethyl ether. Centrifuge and remove all but 1 drop of the aqueous layer. Add 5 ml. of water, stopper, and shake for 30 seconds. Centrifuge and remove all but one drop of the aqueous layer. Wash with two 5-ml. portions of water. Read vitamin A directly.

Fats or oils.[100] Saponify a sample containing not more than 0.66 gram of oil by boiling for 30 minutes with 20 ml. of 95% ethanol and 2 ml. of 50% potassium hydroxide solution under a water-cooled reflux condenser. Cool the flask, take up in 40 ml. of water, and rinse the flask with 30 ml. of hexane. Extract the vitamin A with the hexane used for rinsing, following with 3 additional extractions with 30-ml. portions of hexane. Wash the combined extracts with six 50-ml. portions of water and rinse the separatory funnel with 5 ml. of hexane. Add the rinsings to the extracts and dry with 5-10 grams of anhydrous sodium sulfate. Filter through cotton to separate sodium sulfate and extract the sodium sulfate with six 10-ml. portions of hexane to ensure complete extraction of vitamin A. Dilute to 200 ml. with hexane. Pass through a column of deactivated alumina and read vitamin A directly.

Saponifiable oils.[101] Transfer not more than 1 gram of fat containing not more than 0.15 mg. of vitamin A to an all-glass reflux. Boil with 30 ml. of 85% ethanol and 3 ml. of 50% potassium hydroxide solution for 30 minutes. Cool and add 50 ml. of water. Extract with four 30-ml. portions of redistilled ether. Swirl the combined ether extracts successively with 50-ml. portions of water until the washings do not react to phenolphthalein. Dilute the washed ether extracts to 250 ml.

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[99] Nathan Scott, *Chemist Analyst* **43,** 97 (1954).

[100] D. T. Ewing, L. H. Sharpe, and O. D. Bird, *Anal. Chem.* **25,** 599-604 (1953); Masataro Aoyama, *Kitasato Arch. Exptl. Med.* **24,** 413-5 (1952).

[101] "U. S. Pharmacopoeia," fifteenth ed., pp. 941-2, Mack Publishing Company, Easton, Pennsylvania (1955).

Evaporate a 25-ml. aliquot of the ether extract containing the unsaponifiables to about 5 ml. Then complete evaporation in a stream of inert gas or *in vacuo* at room temperature. Take up in isopropanol having no greater absorbance than 0.05 at 300 mμ or than 0.01 between 320 mμ and 350 mμ. Dilute in that solvent to 3-5 micrograms of vitamin A per ml. to give an absorbance of 0.4-0.8 at 325 mμ. Read directly.

Fish-liver oils.[102] This is substantially the same as the USP method given with the addition of a validity test shown in the procedure.

Alternatively,[103] dissolve a 0.01-gram sample in 5 ml. of 5% potassium hydroxide solution in 95% ethanol and pass in hydrogen gas. Heat for 30 minutes at 70° and cool quickly. Add 10 ml. of benzene and 10 ml. of water. Mix, centrifuge, and decant the benzene layer. Wash the benzene layer with 20 ml. of 5% potassium hydroxide solution in 60% methanol solution followed with water until the extract is completely transparent. Dilute with benzene to an approximate content of 0.0001-0.001 mg. of vitamin A per ml. Dilute 1 ml. to 5 ml. with benzene. Determine by fluorescence.

Vanaspati.[104] Shake a 20-ml. chloroform solution containing the nonsaponifiable fraction from 10 grams of sample with 5 ml. of concentrated hydrochloric acid. Wash twice with 50 ml. portions of water. Wash with 50 ml. of 1% sodium carbonate solution, followed with water until free from alkali. Filter through anhydrous sodium sulfate and wash the residue with chloroform until no vitamin A remains in the sodium sulfate. Read directly.

Milk and milk products.[105] Extract the fat from a 40-ml. sample by shaking successively with 10 ml. of 1:3 ammonium hydroxide, 40 ml. of absolute ethanol, 80 ml. of peroxide-free ether, and 80 ml. of petroleum ether. Let stand for 15 minutes in the dark, remove an aliquot of the supernatant layer, and expel the ethers. To the residue add 1.5 ml. of hydroquinone in absolute ethanol as an antioxidant to permit saponification in air and 15 ml. of 20% ethandic sodium hydroxide solution. To one of two duplicate samples add pure vitamin A to approximately the concentration of the sample. Saponify at 70-80° for 10 minutes and add 8 ml. of aluminum oxide containing 12% of water. Heat until alcoholic fumes are not detectable. Extract with 25 ml. of ethyl ether and wash free of soaps with 5 ml. of water.

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[102] "Official Methods of Analysis of the Association of Official Agricultural Chemists," 8th ed., pp. 812-3, Association of Official Agricultural Chemists, Washington, D.C. (1955); Cf. S. Balasundram, H. R. Cama, P. R. Sundaresan, and T. N. R. Varma, *J. Sci. Ind. Research (India)* **15C**, 23-7 (1956).

[103] A. Fujita and M. Aoyama, *J. Biochem. (Japan)* **38**, 271-5 (1951).

[104] B. R. Roy, *Science and Culture (India)* **20**, 201-2 (1954).

[105] H. Moor, *Milchwissenschaft* **8**, 298-300 (1953).

Evaporate the ether and take up in chloroform so that 2 ml. of solution contains 15-30 units of vitamin A. Develop with antimony trichloride reagent. Alternatively,[106] saponify the sample directly and extract the unsaponifiables with ether.

Butter.[107] Saponify 5 grams by refluxing with 40 ml. of ethanol and 50 ml. of 50% potassium hydroxide solution for 30 minutes. Cool and add 30 ml. of water. Shake with 100 ml. of diethyl ether and separate the water layer. Re-extract the water layer with 60 ml. of diethyl ether. Wash the combined ether extracts with 100-ml. portions of water until the washings no longer react to phenolphthalein. Dry the ether extracts with anhydrous sodium sulfate and dilute to 200 ml. with ether. Evaporate an aliquot of the ether extract under vacuum with carbon dioxide bubbled through. Take up in chloroform and determine with antimony trichloride (Vol. III, pp. 89-90).

Alternatively see page 30.

Feedstuffs containing vitamin A esters extractable with hexane.[108] To a 10-gram sample in an all-glass fat-extraction apparatus without a thimble, add 1 gram of dehydrated alfalfa as a tracer if β-carotene is not to be added later in the procedure. Add 100 ml. of hexane, mark the level, and reflux for 30 minutes. Cover and cool for 10-15 minutes. Add hexane necessary to bring the level to the mark, mix, and allow to settle. Separate vitamin A by chromatography on diatomaceous earth and magnesia.

Blood.[109] Deproteinize a 3-ml. sample with 3 ml. of ethanol and extract the lipid substances by shaking with 6 ml. of hexane. Centrifuge.

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[106] R. A. Wilkinson and J. Conochie, *Australian J. Dairy Tech.* **13**, 32-7 (1958).

[107] R. Sasaki, T. Tsugo, and S. Koyama, *Intern. Dairy Congr. 13th Congr. Hague* **4**, 645-7 (1953).

[108] "Official Methods of Analysis of the Association of Official Agricultural Chemists," 8th ed., p. 814, Association of Official Agricultural Chemists, Washington, D. C. (1955).

[109] S. Bisaz, *Schweiz. med. Wochschr.* **82**, 692-4 (1952).

Evaporate the hexane from a 4.5-ml. aliquot at reduced pressure at 40-50°. Dissolve the yellow oily residue in 2 ml. of hexane and read the carotene at 463 mμ. Evaporate the hexane and take up in 0.5 ml. of chloroform. Develop vitamin A with glycerol dichlorohydrin.

Blood plasma. See page 30.

Margarine.[110] Saponify a 10-gram sample of margarine and extract the unsaponifiable matter. Apply chromatography on bone meal and read directly.

If interfering artificial coloring matter is present,[111] saponify 3-5 grams by boiling for 30 minutes with 20 ml. of 50% potassium hydroxide solution. Extract with four 50-Ml. portions of ethyl ether. Evaporate the combined extracts under nitrogen and take up the residue in 20 ml. of petroleum ether. Add 10 ml. of glacial acetic acid and 1 ml. of 1% acetaldehyde solution. Shake to transfer the coloring matter to the acetic acid layer. Separate the petroleum ether layer and evaporate to dryness. Take up the residue in chloroform for determination by antimony trichloride or in isopropanol for direct reading.

Hens eggs. See page 29.

Procedure—*Direct reading.*[112] For this determination to be valid, the sample must pass the colorimetric validity test. To prepare the antimony trichloride solution, dissolve 113.4 grams of antimony trichloride in 300-400 ml. of redistilled chloroform. Add about 5 grams of calcium chloride, mix, and filter while hot. Dilute to 500 ml. with chloroform.

Evaporate the ether from a portion of the unsaponifiable extract of the sample in a stream of inert gas. Dilute with chloroform to give concentrations that have, after addition of reagent, absorbance of approximately 0.2. Add antimony trichloride reagent, and read within a few seconds at the point of maximum absorbance.

The following formula must not be less than 1 nor more than 1.3 for the determination to be valid.

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[110] J. W. Lord and P. M. Bradley, *Analyst* 80, 429-39 (1955); Cf. Lawrence Rosner and Henrietta Kan, *J. Assoc. Offic. Agr. Chemists* 37, 887 (1954); B. Barnholdt, *Vitaminforsch.* 28, 365-86 (1958).

[111] Koji Naito and Takajiro Mori, *Vitamins (Japan)* 8, 17-20 (1955).

[112] "Official Methods of Analysis of the Association of Official Agricultural Chemists," 8th ed., pp. 813-4, Association of Official Agricultural Chemists, Washington, D. C. (1955); Cf. Louis Korr, *Chemist Analyst* 42, 15 (1953).

$$\frac{C_u \times C_s}{(C_{u+s} - C_u) \times W \times (\text{microgram vitamin A/gram sample})}$$

where

C_s = micrograms of vitamin A in each ml. of the final standard solution in chloroform as calculated from the weight of standard saponified

C_u = micrograms of vitamin A in each ml. of the final solution of sample as calculated from the blank-corrected absorbance and calibration curve

C_{u+s} = micrograms of vitamin A in the solution of reference standard + sample as calculated from the standard curve

W = sample units represented in each ml. of final chloroform dilution for assay.

Evaporate a 10-ml. aliquot of the diethyl ether solution of the unsaponifiable extract to about 2 ml. Blow off the remaining ether in a stream of nitrogen at room temperature. Take up in isopropanol or absolute ethanol to give an expected absorbance of 0.4-0.8 at 325 mμ. Read at 310, 325, and 334 mμ. Calculate the vitamin A as follows:

$$\text{Corrected A} = 7\,A_{325} - 2.625\,A_{310} - 4.375\,A_{334}$$

$$\text{Micrograms of vitamin A} = (5.7/LW)\text{ corrected A}$$

W = decimal fraction of sample in 1 ml. of the solution read;

L = length of cell in cm.

*By dehydration with toluene-*p*-sulfonic acid as catalyst.* To prepare the catalyst reflux 15 mg. of toluene-*p*-sulfonic acid monohydrate with 100 ml. of redistilled benzene until dissolved. Distil or boil off 10 ml. of solvent to drive out moisture. Allow the solution to cool, protected from moisture. Readjust the volume to 100 ml. with dry benzene and activate before use by repeating the distillation.

Dilute a sample of unsaponifiable extract containing vitamin A in dry benzene to contain 5-50 international units per ml. Mix 1 ml. of this benzene solution with 4 ml. of catalyst. After 1 minute neutralize with 5 ml. of 2% sodium hydroxide solution or shake with 1 gram of sodium carbonate for 1 minute. Clarify by centrifuging or settling and read at 390 mμ against a mixture of 1 volume of the unsaponifiable fraction in benzene and 4 volumes of benzene. Convert the readings to the amount of vitamin A in international units as follows:

$$\text{Amount of vitamin A} = E_D/0.0122$$

where E_D is the increase in optical density caused by dehydration and 0.0122 is the optical density increase corresponding to 1 international unit of vitamin A which is equivalent to an extinction coefficient of 2030.

By glycerol dichlorohydrin.[113] To determine the antimony trichloride content add to 1 ml. of glycerol dichlorohydrin which has been redistilled at 100 mm. of mercury and 65-70°, 20 ml. of 10% aqueous potassium sodium tartrate solution and 2 grams of sodium bicarbonate. Mix and titrate with 0.1 *N* iodine. Each ml. of 0.1 *N* solution equals 11.4 mg. of antimony trichloride. Adjust the antimony trichloride to 25 mg, per ml.

Dissolve a 0.01-gram sample in 5 ml. of chloroform. Add a 1-ml. aliquot to 2 ml. of glycerol dichlorohydrin, containing 0.01-0.05 gram per ml. of antimony trichloride, and 0.1 ml. of concentrated hydrochloric acid. Mix and read the purple color at once at 550 mμ.

By perchloric acid.[114] Follow the procedure for carotene alone page 30 ending with "Add 2 ml. of 1:1 perchloric acid." Read the purplish-red color at 525 mμ within 15 minutes after addition of perchloric acid.

By fluorescence. Match an aliquot in benzene containing 0.0001-0.001 mg. of vitamin A per ml. of benzene against benzene containing the vitamin A standard solution.[113]

TOCOPHEROLS, VITAMIN E

On treatment with nitrous acid, alcoholic solutions of β-, γ-, and δ-tocopherols form yellow nitroso derivatives because of the presence of an unsubstituted position ortho to the phenolic group.[115] When treated with alkalies they form red but turbid solutions. On dilution and extraction with petroleum ether they give stable yellow solutions.

Peroxide-free dimethoxyethane is a preferable inert medium in determination of γ-tocopherol. It is both a solvent for γ-tocopherol and is miscible with aqueous reagents. In the presence of ethanol, interfering absorption bands are observed which were probably due to the presence of ethyl nitrite, formed by the action of nitrous acid on ethanol. The nitroso-γ-tocopherol complex is extracted with isooctane to eliminate interfering colors from the dimethoxyethane blank and sample solutions containing the reagents. All operations should be performed under subdued artificial light.

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[113] A. Fuijita and M. Aoyamo, *J. Biochem. (Japan)* **38**, 263-9 (1951).

[114] Peter Flesch, *Proc. Soc. Exptl. Biol. Med.* **84**, 148-9 (1953).

[115] Barbara Hinderer Polister, *Anal. Chem.* **26**, 407-8 (1954).

When 5 ml. of absolute ethanol solution of tocopherols is boiled with 1 ml. of concentrated nitric acid (Vol. III, p. 93) for 5 minutes, a maximum coloration is obtained.[116] After holding for 40 minutes at room temperature, teh color is linear over the range of 0.4-4 mg. at 466 mμ. This oxidation is also applied in 3:7 chloroform-ethanol.[117]

Vitamin E is measured in mixtures of vitamins A, B_1, B_6, C, E, and niacin by reduction of ferric chloride and measurement of the ferrous ion with 2,2′,-bipyridine (Vol. III). The mixture is first saponified and vitamin E extracted with diethyl ether.[118] Oxidation products of α-tocopherol and phenolic antioxidants show the same color. The oxidation products are removable by chromatographing on alumina, the antioxidants by extraction with petroleum ether-diethyl ether.[119] If carotenoids are present, the correction for them may amount to 2-4 times the content of α-tocopherol.[120]

This method is applicable as an index of the adulteration of butter oil. Carotenes, vitamin A, and synthetic food colors such as 1-phenylazo-2-naphthylamine (Oil Yellow AB) and 1-*o*-tolylazo-2-naphthylamine (Oil Yellow OB) interfere. The food colors and vitamin A are removed by extraction with 3:2 sulfuric acid and the carotene content is estimated at 440 mμ after the sulfuric acid extraction. The corrected tocopherol content is with ±2% of the quantities present.[121]

In the presence of glacial acetic acid, phosphomolybdic acid reacts with α-tocopherol to produce a yellow to green color which obeys Beer's law at the level of 0-0.025 mg. of α-tocopherol per ml.[122] The specificity of the reaction depends on the preparation of the reagent. Sensitivity of the reaction is increased at elevated temperatures.[123] Steroids with α-betol side chains react with the reagent. β-Carotene, vitamin A, calciferol, cholesterol, and carotenoids do not interfere.[124]

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[116] Toku Tetsuka, *Vitamins* (*Japan*) **8,** 352-5 (1955).

[117] Kazimiera Pietroniuk, *Chem. Anal.* **2,** 77-90 (1957).

[118] L. C. Via and M. Giannini, *Farmaco* (*Pavia*) *Ed. prat.* **11,** 229-34 (1956).

[119] G. Katsui, Y. Matsuaka, and T. Tetsuka, *Vitamins* (*Kyoto*) **8,** 352-5 (1955).

[120] B. G. Savinov and G. M. Lushchevskaya, *Ukrain. Khin. Zhur.* **16,** 370-82 (1950); **18,** 540-6 (1952).

[121] J. H. Mahon and R. A. Chapman, *Anal. Chem.* **26,** 1195-8 (1954).

[122] P. P. Nair and N. G. Magar, *Indian J. Med. Research* **42,** 577-84 (1954).

[123] Harris Rosenkrantz, *J. Biol. Chem.* **224,** 165-74 (1957).

Tocopherols as the vitamin E, acetate are determined by deacetylation and read against the unacetylated compound.[125] There is no interference by vitamins A, B_1, B_6, C, and niacin under the conditions of test.

Up to 1 mg. of vitamin E can be estimated fluorimetrically. Vitamins A, D, and K interfere but light-exciting and fluorescence-selecting filters permit the determination of vitamin E in multivitamin preparations. Large amounts of inhibiting substances are removed by condensation with *o*-phenylenediamine followed by chromatography on alumina,[126] or extraction with petroleum ether.[127]

The method does not distinguish between α-, β-, and γ-tocopherols. Small amounts of vitamin A and other fat-soluble reducing substances can be tolerated, larger amounts by use of an internal standard.[128] Beer's law is followed for 0.00001-0.04 mg. per ml.

The interference of autoxidizing methyl esters of fatty acids with determination of α-tocopherol by bipyridyl or direct reading in the ultraviolet is eliminated by dissolving the sample in petroleum ether and shaking with 4:1 sulfuric acid.[129] If oxidized fat is to be removed for spectrophotometric reading, saponify in ethanol-petroleum ether protected from oxygen by pyrogallol.

Samples—*Blood plasma and serum.*[130] Centrifuge a whole blood sample for 20 minutes. To 1-2 ml. of serum or plasma add 0.5 ml. of 5% sodium hydroxide solution and shake well to saponify any esters of vitamin E present. Let stand for 6 hours and dilute with 10 ml. of water. Extract twice with 5-ml. portions of petroleum ether and wash the combined extracts with water until the washings are alkali-free as indicated with phenolphthalein. Evaporate the petroleum ether under partial vacuum. Develop with phosphomolybdic acid.

Alternatively,[131] mix a 2-ml. sample with 10 ml. of 95% ethanol. Extract with 5 ml. and 5 ml. of benzene. Combine the benzene extracts and dry with anhydrous sodium sulfate. Pass through a column of diatomaceous earth to remove interferences. When the vitamin E has been washed through the column, evaporate the benzene solution to dryness. Take up the residue in 95% ethanol and develop with ferric chloride and α-α'-bipyridine.

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[124] P. P. Nair and N. G. Magar, *Indian J. Med. Research* 42, 577-84 (1954); *J. Biol. Chem.* 220, 157-9 (1956).

[125] L. Campaioli and M. Giannini, *Farmaco (Pavia) Ed. prat.* 11, 229-34 (1956).

[126] G. Katsui and Y. Matsuoka, *Vitamins (Kyoto)* 11, 369-74 (1956).

[127] M. Kafler, *Helv. Chim. Acta* 25, 1469-74 (1942).

[128] Daniel E. Duggan, *Arch. Biochem. and Biophys.* 84, 116-22 (1959).

[129] H. J. Lips, *J. Am. Oil Chemists' Soc.* 33, 426-8 (1956).

[130] P. P. Nair and N. G. Magar, *J. Biol. Chem.* 220, 157-9 (1956).

[131] A. M. Epel'baum and G. M. Lushchevskaya, *Vitaminy, Akad. Nauk Ukr. S.S.R., Inst. Biokhim.* 3, 95-100 (1958).

Tissue. Homogenize a 2.5-gram sample with 5 ml. of 1:1 ethanol. Centrifuge to separate the clear upper layer. Extract with 2-3 ml. of isooctane. Dry 1 ml. of the organic layer under nitrogen and develop with phosphomolybdic acid.

Tissue extracts. Homogenize a 250-mg. sample in 5 ml. of 50% ethanol. Centrifuge to separate the clear upper layer. Treat as for tissue from "Extract with 2-3 ml. of isooctane."

Mixtures of vitamins A, B_1, B_6, C, E, and niacin. Evaporate a sample below 70° in a stream of nitrogen. Add 4 ml. of 5% pyrogallol solution in ethanol and saponify with 1 ml. of potassium hydroxide solution containing 4 grams of potassium hydroxide in 2.7 ml. of water. Heat for 5 minutes at 100°, add 8 ml. of water, and extract with diethyl ether. Dilute the extract to 250 ml. Dilute a 2-ml. aliquot of the ether solution with petroleum ether to 10 ml. Develop with ferric chloride and 2,2′-bipyridine.

Butter oil.[132] Heat a cubed sample at 60° in an oven for the least possible time to separate the butter oil. Decant the oil and filter at 35°. The filtrate should be clear. Dissolve 2.5 grams in petroleum ether and dilute to 50 ml. with petroleum ether. Shake with 10 ml. of 3:2 sulfuric acid for 20 seconds to extract the synthetic food colors, Oil Yellow AB, and Oil Yellow OB, and vitamin A. Let stand for 15 minutes and remove the pink acid layer. Wash the petroleum ether for a few seconds with two 25-ml. portions of water. Develop with ferric chloride and 2,2′-bipyridine.

Fresh green plants.[133] Extract 2-3 grams of crushed sample with 15-25 ml. of ethanol followed by 15-25 ml. of benzene. Shake with 1.5-2.5 ml. of 50% potassium hydroxide solution for 20 minutes to saponify. Add water, discard the aqueous layer, and filter the benzene layer through anhydrous sodium sulfate. Determine carotenoids from the yellow color in the benzene layer (page 30). Evaporate the benzene and reflux the residue with 5 ml. of absolute ethanol. Develop with nitric acid.

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[132] J. H. Mahon and Ross A. Chapman, *Anal. Chem.* **26**, 1195-9 (1954).

[133] B. G. Savinov and G. M. Lushchevskaya, *Ukrain. Khim. Zhur.* **16**, 370-82 (1950).

Procedure—*By nitric acid.* To 5 ml. of sample in absolute ethanol add 1 ml. of concentrated nitric acid. Reflux for 3 minutes to develop a rose color. Read at 465 mμ and, if present, correct for carotenoids.

γ-Tocopherol nitrous acid. To 5 ml. of solution containing 0.1-1.2 mg. per ml. of γ-tocopherol, free from β- and γ-tocopherols, in freshly distilled, peroxide-free dimethoxyethane, add 0.2 ml. of glacial acetic acid. Mix and add rapidly 3 ml. of 2% sodium nitrite solution. Swirl vigorously for 5 seconds and let stand for exactly 60 seconds. Add 2 ml. of 20% potassium hydroxide solution and mix. Add 10 ml. of water, a small amount of anhydrous sodium sulfate to facilitate the separation of the layers following extraction, and 10 ml. of isooctane. Stopper, shake vigorously for 30 seconds, and allow to settle. Read the isooctane layer at 350-450 mμ in the visible region and 280-340 mμ in the ultraviolet. Subtract a reagent blank. The same reaction report β- and γ-tocopherols but not necessarily at the same maximum. The details are valid only for d-γ-tocopherol.

By phosphomolybdic acid. To the sample in petroleum ether containing 0.002-0.005 mg. of vitamin E, add 1 ml. of 0.394% phosphomolybdic acid in glacial acetic acid. After exactly 15 minutes, dilute with 3 ml. of absolute ethanol to a known volume. Shake and read at 725 mμ against water. With samples containing up to 0.15 mg. of α-tocopherol, use 2 ml. of 0.2% phosphomolybdic acid solution in glacial acetic acid.

By ferric chloride and 2,2′-bipyridine. To 10 ml. of sample containing 0.01-0.08 mg. of tocopherol in petroleum ether add 0.2 ml. of absolute ethanol to absorb any entrained water. If the solution is still turbid, repeat. Read at 440 mμ against petroleum ether treated the same as the sample to determine the carotene content.

If there is no carotene present, start the determination at this point. To 10 ml. of sample containing 0.01-0.08 mg. of tocopherol in petroleum ether, add 5 ml. of 0.04% ferric chloride solution in ethanol and 5 ml. of 0.4% 2,2′-bipyridine solution in ethanol. After 6 minutes from the time the ferric chloride is added, add 2 ml. of water and invert twice. Draw off the alcohol layer after 10.5 minutes from the time the ferric chloride reagent is added. Filter and read after 1.5 minutes at 515 mμ against a reagent blank.

Calculate the absorbancy corrected for carotene as follows:

$$A_{515} - (0.26 \times A_{440}) = \text{corrected absorbancy.}$$

The corrected absorbancy divided by 0.00506 yields the concentration of tocopherol expressed as α-tocopherol per aliquot used.

Acetylated to avoid interference of carotenoids. Evaporate an appropriate portion of sample below 70° in a stream of nitrogen. Add 4 ml. of 5% pyrogallol in 95% ethanol and 1 ml. of 60% potassium hydroxide solution. Heat at 100° for 5 minutes to saponify and add 8 ml. of water. Extract with 25, 25, and 25 ml. of ether. Dilute the combined extracts to 250 ml. with ether. Dilute 2 ml. to 10 ml. with petroleum ether. From there on the comparison is of the acetylated and nonacetylated tocopherols to avoid interference of carotenoids.

Evaporate two 1-ml. portions of sample under nitrogen at 70°. To one add 0.2 ml. of acetic anhydride and 0.2 ml. of pyridine and heat for 10 minutes at 70°. Evaporate unreacted reagent under nitrogen. To each tube add 10 ml. of a reagent containing 4% of 0.2% ferric chloride in ethanol, 4% of 0.5% α,α′-bipyridine, 40% of benzene, and 52% of anhydrous ethanol. After 10 minutes in the dark read both the unacetylated and the acetylated tocopherol at 520 mμ. The difference in readings is the measure in terms of pure α-tocopherol.

Fluorometric after nitration.[134] To a 10-ml. sample in absolute ethanol, containing up to 1 mg. of tocopherol, add 2 ml. of concentrated nitric acid and boil for 5 minutes. Take up in 15 ml. of low-boiling petroleum ether, dilute with water, and shake. Drain off the aqueous layer, wash the petroleum ether once with water, and evaporate at 100°. To the residue add 5 ml. of 1% *o*-phenylenediamine solution in glacial acetic acid to condense interferences, and heat at 100° for 1 hour. Cool, take up with 15 ml. of low-boiling petroleum ether, dilute with water, and shake. Remove the aqueous layer and wash the petroleum ether successively with water, 1:500 hydrochloric acid, 0.1% sodium hydroxide solution, and water. Remove the petroleum ether by evaporation and take up in 10 ml. of methanol. Compare with standards in a fluorometer.

Direct fluorimetry. Plasma. To prepare the hexane, wash hexane with concentrated sulfuric acid until the acid layer is essentially colorless. Wash with dilute alkali, and then dry. Pass through a column of silica gel of sufficient length to retain the dark yellow band. Distil.

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[134] M. Kafler, *Helv. Chim. Acta* **25**, 1469-74 (1942).

To a 2-ml. sample, add 2 ml. of water and 4 ml. of absolute ethanol. Mix and add 8 ml. of hexane. To prepare the internal standard, to another 2 ml. of sample, add a small volume of an alcoholic solution containing equimolar quantities of α-tocopherol and tocopheryl acetate in the area of 0.05 micromole. Shake the internal standard thoroughly. Add 2 ml. of water and 4 ml. of absolute ethanol and mix. Add 8 ml. of hexane. Agitate both the sample and standard for 10 minutes and centrifuge lightly.

Free tocopherol. Dilute a 1 ml. aliquot of the clear organic phase with 3 ml. of absolute ethanol and measure its fluorescence at 340 mμ upon activation at 295 mμ. Compare with the internal standard similarly treated.

Free and esterified tocopherol from tocopheryl acetate. Evaporate a 4-ml. portion of the clear organic phase to dryness under nitrogen. Draw the nitrogen through the residue for several more minutes to insure removal of the solvent. Redissolve in 4 ml. of hexane and cool in ice.

To hydrolyze the tocopheryl acetate to the alcohol, add 1 ml. of a lithium aluminum hydride reagent prepared by shaking 20 ml. of cold, freshly distilled ether with 200-300 mg. of finely powdered lithium aluminum hydride and filtering through glass wool. Shake occasionally for 5 minutes and carefully add 1 ml. of cold absolute ethanol. A vigorous evolution of gas at this point indicates cleavage of the tocopherol esters. Absence of the gas evolution indicates insufficient lithium aluminum hydride added. Add 1 ml. of 1:350 sulfuric acid, stopper, and shake occasionally for 5 minutes. Dilute an aliquot of the organic phase with 3 volumes of absolute ethanol and measure the fluorescence at 340 mμ upon activation at 295 mμ. Compare with the similarly treated internal standard.

CHAPTER 4

PHENOLS

Confusion in the determination of phenol or phenols may be semantic. To a chemist phenol means a chemical individual characterized by the usual properties of melting point, vapor pressure, and physical properties of derivatives. Phenols may mean anything with an aromatic hydroxyl group, may extend far beyond what is ever thought of as a phenol, a complex dye having one such group on a large molecule. The result is that general methods for phenol and phenols can be and are thoroughly confusing. And the confusion cannot end there.

Table 4. Absorption Spectra of Weak Organic Acids in Neutral and Alkaline Dimethylformamide and Alkaline Ethanol

	λ_{max} ($\epsilon \times 10^{-3}$)		
Compound	DMF	*Alk.* DMF[a]	*Ethyl alcohol*[b]
Phenol	275 (1.8)		280 (2.0)
	281 (1.5)	310 (2.0)	290 (2.03)
1-Naphthol	298 (5.40)	356 (5.60)	336 (7.50)
4-Nitro-*N*-perfluorobutyrylaniline	299 (14.0)[c]	401 (20.7)	358 (12.7)
4-Trifluoroacetylaminoazobenzene	338 (27.0)	395*s* (14.0)	375 (17.7)
		420 (15.6)	
1-Anthrol	395Fs (4.0)[d]	482 (3.95)	448 (4.20)
2-Hydroxyanthraquinone	375 (2.9)	548 (5.3)	482 (5.3)
Isatin	419 (0.87)	550 (0.71)	513 (0.63)
Acetophenone-4-nitrophenylhydrazone	413 (30.4)	553 (48.0)	529 (21.0)
1-Hydroxyanthraquinone	402 (5.5)	561 (5.90)	502 (5.60)
Cinnamaldehyde-4-nitrophenylhydrazone	418 (44.0)[c]	575 (65.8)	554 (38.4)
1,3-Bis(4-nitrophenyl)triazene	409 (38.6)	580 (72.0)	532 (56.8)
2-Anilino-3-chloro-1,4-naphthoquinone	482 (4.50)	600 (5.40)	563 (4.60)
4′-Nitro-4-hydroxyazobenzene	385 (26.4)	605 (26.4)	510 (35.0)
4-Nitrobenzaldehyde-4-nitrophenylhydrazone	428 (42.2)	667 (63.0)	600 (41.8)
4-(4′-Nitro-1′-naphthylazo)-1-naphthol	480 (20.0)	700 (73.0)	660 (35.4)

[a] Dimethylformamide containing 2% of 10% aqueous tetraethylammonium hydroxide.

[b] 95% ethyl alcohol containing 2% of 10% aqueous tetraethylammonium hydroxide.

[c] In 95% ethyl alcohol.

[d] Fs, fine structure.

TABLE 5. PER CENT IONIZATION OF WEAK ACIDS IN BASIC SOLVENTS[a]

Weak acid	*Per cent ionization*[b] *Dimethylformamide*	*Acetone*	*1-Butanol*	*2-Methoxyethanol*	*Methanol*
1,3-Di-*p*-nitrophenyltriazene	100	100	100	100	100
4-Nitrobenzaldehyde-4-nitrophenylhydrazone	100	100	100	100	20
3-Nitrobenzaldehyde-4-nitrophenylhydrazone	100	100	100	100	5
4-Diethylaminobenzaldehyde-4-nitrophenylhydrazone	100	100	100	~50	0
2-Nitrobenzaldehyde-2-nitrophenylhydrazone	100	100	10	0	0
4-Nitrobenzaldehyde-2-nitrophenylhydrazone	100	100	5	0	0
4-Phenylazodiphenylamine	100[c]	35	0	0	0
4-Phenylazoaniline	~40	0	0	0	0

[a] All solutions contain 2% of 10% tetraethylammonium hydroxide in water.
[b] Determined by ultraviolet visible absorption spectroscopy.
[c] With addition of 3% water, ionization was 80%.

Often for all practical purposes there is no reason to distinguish between phenol, cresols, xylenols, and more complex bodies which have the same desirable or undesirable properties. With that enigmatic confusion we introduce the aliphatic hydroxyl compounds.

The absorbancy of phenols or acids is increased and the wave length of maximum absorbancy is increased by conversion to an alkaline salt.[1] The effect is also altered by the basicity of the solvent. Conversely hydroxylic solvents lower the wave length and its intensity.

The effect of basic solvents is shown in Table 4. The effect of the selection of solvent is shown in Table 5. The effect of the addition of hydroxylic solvents is shown in Table 6. The possible solvents and their effect on the same solute is shown in Table 7. The basic principle therefore spells out, subject to solubility, the methods of increasing or decreasing the absorbancy, increasing or decreasing the location of the maximum absorbancy.

Various phenols when reacted with titanium sulfate are read spectrophotometrically. The maxima vary: *m*-aminophenol 417 mμ, *p*-aminophenol 418 mμ, *p*-bromophenol 473 mμ, *p*-chlorophenol 465 mμ, 2,4-di-

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[1] E. Sarvicki, T. R. Hauser, and T. W. Stanley, *Anal. Chem.* 31, 2063-5 (1959).

Table 6. Effect of Water on Absorption Spectra of Some Anions in Alkaline Dimethylformamide[a]

	λ_{max} ($\epsilon \times 10^{-3}$)				
Compound	0% H_2O	25% H_2O	50% H_2O	75% H_2O	95% H_2O
4-Nitrophenol	426 (27.6)	415 (22.0)	410 (20.0)	406 (18.0)	401 (16.6)
4-Hydroxychalcone	481 (43.6)	454 (34.0)	444 (30.8)	433 (29.0)	425 (28.0)
2-Methyl-4-hydroxyazobenzene	450*s* (26.8)[b] 485 (28.2)	440*s* (18.6) 461 (20.0)	425 (22.6) 443*s* (22.0)	415 (22.8) 440*s* (22.0)	408 (22.0) 435*s* (20.4)
3-Methyl-4-hydroxyazobenzene	489 (41.4)	475 (29.6)	425*s* (22.0) 453 (26.2)	425*s* (22.0) 450 (25.2)	425*s* (22.0) 446 (24.0)
2-Hydroxyanthraquinone	530 (6.2)	502 (5.0)	485 (4.8)	475 (4.7)	470 (4.6)
6-Hydroxy-5,12-naphthacenedione	534 (11.2)	530 (9.6)	525 (9.4)	521 (9.0)	. . .
4-(4′-Acetylphenylazo)-1-naphthol	586 (60.0)	. . .	. . .	. . .	548 (38.8)
4′-Nitro-4-hydroxyazobenzene	605 (60.0)	535 (39.2)	511 (34.2)	498 (31.4)	486 (30.4)
4-(4′-Nitrophenylazo)-1-naphthol	650 (83.0)	629 (63.6)	612 (55.2)	600 (45.4)	581 (36.0)
4-Nitrobenzaldehyde-r-nitrophenylhydrazone	667 (63.0)	625 (53.4)	607 (38.2)	. . .	. . .
4-(4′-Nitro-1′-naphthylazo)-1-naphthol	700 (73.0)	700 (51.2)	680 (38.5)	650 (31.6)	612 (26.4)

[a] All solutions contain 2% of 10% aqueous tetraethylammonium hydroxide in addition to the given percentage of water.

[b] *s* = Shoulder.

chlorophenol 490 mμ, 5-hydroxy-1,3-dimethylphenol 453 mμ, *p*-nitrophenol 451 mμ, *p*-tert-butylphenol 469 mμ, *p*-phenylphenol 482 mμ, pyrogallol 460 mμ, and 5-hydroxy-1,3-dimethylphenol 453 mμ.

While phenols are considered phenol is a more limited subject. After chromatographic separation from cresols, xylenols, and ethyl phenol, phenol is read directly in the ultraviolet, page 85. Phenol in cigarette smoke is converted to the azo compound of *p*-nitroaniline and separated from cresols and guaiacol by paper chromatography, page 89. Reaction with titanium sulfate gives a maximum absorption at 455 mμ, page 93.

Phenolic acids and catecholic phenols are determined in urine by ferrous ion, page 91. After separation of 2-ethylphenol along with xylenols from cresols and phenol by chromatography it is calculated by

TABLE 7. ABSORPTION SPECTRA OF 4′-NITRO-4-HYDROXYAZOBENZENE IN ALKALINE SOLUTION

λ_{max}	$\epsilon \times 10^{-3}$	*Solvent*[a]
605	60.0	*N,N*-Dimethylformamide
605	49.0	Pyridine
600	58.8	*N*-Methyl-2-pyrrolidone
590	55.6	Dimethylsulfoxide
582	48.0	*N,N*-Dimethylacetamide
568	40.8	2-Pyrrolidone
565	51.0	Acetone
560	40.4	Dimethylaniline
560	40.0	Aniline
560	41.4	Nitropropane
560	38.8	Dimethylmalonate
555	44.2	Nitromethane
543	39.0	Xylene
540	37.0	*n*-Butyl alcohol
538	35.0	Chloroform
520	37.8	Isoamyl alcohol
520	35.4	2-Methoxyethanol
520	33.6	Formamide
515	35.0	*n*-Propyl alcohol
517	32.0	Benzyl alcohol
510	35.0	Ethyl alcohol
495	26.0	2,2,2-Trichloroethano
490	42.0	Ethylene glycol
490	31.0	Methyl alcohol
485	30.3	Water
450	24.0	2,2,2-Trifluoroethanol

[a] All solutions contain 2% of a 40% methanolic solution of *N*-benzyltrimethylammonium methoxide.

reading in the ultraviolet, page 86. The *m*- and *p*-ethylphenols are isolated chromatographically and read in the ultraviolet, page 86. By reaction with titanium sulfate, page 93, a maximum absorption is obtained for *p*-ethylphenol at 473 mμ. The maximum fluorescence of phenylephrine at pH 1 is at 305 mμ.[2]

o-Cresol in cigarette smoke is converted to the azo compound of *p*-nitroaniline. Thereafter it is separated from *p*-cresol, guaiacol, and phenol by paper chromatography, page 89. It is also separated from samples chromatographically accompanied by 3,4-xylenol. It is then read in the ultraviolet (Cf. Vol. III, p. 120). After reaction with titanium sulfate the maximum absorption is at 455 mμ, page 93. Similarly, *m*-cresol in cigarette smoke is converted to the azo derivative of *p*-nitrophenol and separated from phenol, *o*-cresol, and guaiacol by paper chromatography, page 89. *m*-Cresol can also be determined in the presence of *p*-cresol, *o*-cresol, and phenol by nitration in cold acetic acid and measurement of the color formed against the sample solution as a blank.

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[2] S. Udenfriend, D. E. Duggan, B. M. Vasta, and B. B. Brodie, *J. Pharmacol. Exptl. Therap.* **120,** 26-32 (1957).

Separation chromatographically gives *o*-cresol and 3,4-xylenol. By reading in the ultraviolet each is calculated, page 86. 2:3-, 2:4-, and 2:5-xylenols are separated from cresols and phenol by chromatography. Thereafter they are read in the ultraviolet along with 2-ethylphenol. Each is then calculated by simultaneous equations, page 88. Chlorocresol is at page 490.

While there are limited methods cited for noradrenaline, also known as *l*-α(aminomethyl)3-4-dihydroxybenzyl alcohol, *l*-arterenol, and *l*-norepinepherine, they are so closely allied to those for adrenaline that they have been incorporated into that topic, page 111. And as naturally occurring it is usually a minor ingredient associated with adrenaline. When the methylated derivatives, metanephrine and normetanephrine, are oxidized and rearranged in alkaline solution in the presence of oxygen, the fluorescence is like that of adrenaline and noradrenaline.[3]

Phloroglucinol reacts with *p*-dimethylaminobenzaldehyde, as does resorcinol also, page 109. When reacted with titanium sulfate the maximum absorbance is at 460 mμ, page 93. A method for 8-hydroxyquinoline (Vol. III, pp. 151-2) depends on the reaction with diazobenzene-sulfonic acid in a solution buffered at 9.0-9.5 to produce a yellow-orange to red-orange color which is read at 425 mμ.[4]

Comparison has been made of phenol, *o*-, *m*-, and *p*-cresol, and mixed tar acids from coal-gas tar.[5] In determination by *p*-nitroaniline, page 89, at 517 mμ the color was more stable than as nitrosophenols. The best wave length for determination as nitrosophenols was at 420 mμ. In both precision and time the most desirable method was by 4-aminoantipyrine at 510 mμ, page 91.

Determination of 2,2′-methylenebis(3,4,6-trichloro) phenol, hexachlorophene, G-11, appears under halogen compounds, page 498. Phenolsulfonic acid appears on page 476.

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[3] A. Bertler, A. Carlsson, and E. Rosengren, *Clin. Chim. Acta* **4**, 456-7 (1959).

[4] Z. Bultas *et al., Českoslov. farm.* **2**, 80-4 (1953).

[5] Satoyoshi Ozaki, *Bunseki Kagaku* **7**, 275-83 (1958).

A method for either α- or β-naphthol fluorimetrically is given under α-naphthol (Cf. Vol. III, pp. 146-7). Alternatively precipitate β-naphthol from α-naphthol in 50% methanol by adding sodium nitrite and hydrochloric acid. Filter and add more sodium nitrite to the filtrate to develop a red color.[6] When reacted with titanium sulfate β-naphthol shows a maximum absorption at 435 mμ, page 93.

PHENOLS

The subject here is class reactions for phenols (Vol. III, pp. 104-120). The simplest method is by reading an alkaline solution of phenols in the ultraviolet. Unless the structure of the phenols is reasonably uniform this introduces a serious problem of standards, which may be approximate.

Free phenols in polyoxyethylene phenyl ethers are read directly at 288 mμ in an alkaline solution of the sample.[7] Beer's law is followed for 0.1-2 mg. of phenol per 100 ml. Over the range of 0.0008-0.8% the accuracy is to about 5%.

To abbreviate the technic of reading phenols in the ultraviolet, one depends on the shift of the spectra toward longer wave lengths when ionized.[8] A practical application is determination of all phenolic bodies in waste waters[9] where ten parts per billion may be significant. In operation interfering oil is extracted with carbon tetrachloride at pH 12 and phenols are extracted with tributylphosphate at pH 5. The resulting concentrate is then read at pH 5 and 12 to give a bathochromic difference. Materials insensitive to pH change then do not interfere.

Since the individual phenols in waste water are not identified for analysis an average absorptivity value must be employed for analyzing unknowns. Beer's law is not followed. Naphthols and dihydric phenols interfere appreciably, thiophenol slightly. The interference from aromatic nitrogen compounds and carboxylic acids is negligible. Phenolsulfonic acids do not interfere. The method is sensitive to 0.1 ppm. of phenols. If the amount of extraneous compounds in the extract is low, longer cells can be used to improve accuracy. Inorganic salts must be absent from the reagent to prevent the salting out of water in the tributyl phosphate extracts.

Phenols, cresols, xylenols, and ethyl phenols are separated by chromotography and determined in the ultraviolet. A cyclohexane-water or cyclohexane-methyl Cellosolve solvent system is used, the latter providing a more detailed separation into 6 groups identified under the procedure.[10]

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[6] L. Nicolas and R. Burel, *Chim. anal.,* 38, 316-21 (1956).

[7] C. F. Smullin and F. P. Wetterau, *Anal. Chem.* 27, 1836-8 (1955).

[8] H. W. Lemon, *J. Am. Chem. Soc.* 69, 2998-3000 (1947).

[9] L. J. Schmauch and H. M. Grubb, *Anal. Chem.* 26, 308-11 (1954).

Mixtures of ortho and para isomers of phenols are determined in cyclohexane since para substitution causes a spectral shift in the position of the major peaks of the curves of the two isomers.[11] Less than 1% of one isomer in another cannot be detected by this method. The technic is applicable for 5-95% of one isomer to give a percentage composition within 1%. The best wave length for analysis varies with the specific ortho and para isomers to be analyzed but the general range is 285 ± 2 and 273 ± 2 $m\mu$. If any meta isomer is present it is likely to be included with the ortho isomer because their spectra are nearly identical.

In the analysis of phenols in water and waste waters, the 4-aminoantipyrine procedure (Vol. III, p. 119) is the fastest, most precise, and most accurate even in parts per billion range when compared with 2,6-dibromoquinone chloromide (Vol. III, p. 118), nitrosophenol (Vil. III, p. 119), ultraviolet, or infrared readings.[12] When the coupling position is blocked the ultraviolet method is preferable.

For the 4-aminoantipyrine method to be applicable at least one free phenolic group must be present. The position para to the phenolic group must be unsubstituted or substituted by halogen, carboxyl, sulfonic acid, hydroxyl, or methoxyl which are expelled by the reaction. The para position must not be substituted by alkyl, aryl, nitro, benzyl, nitroso, or aldehyde groups which block the reaction. The reaction is carried out in a borate buffer in preference to the previously used ammonium hydroxide-ammonium chloride buffer which was affected by variations in ionic strength and had a lesser buffering capacity. Potassium ferricyanide is retained as the oxidizing agent.[13]

Phenols are extracted from acylic monomers with sodium hydroxide. The color, which develops immediately on addition of the reagents, is stable for 1 hour. The absorption peak is at 510 $m\mu$. Up to 1% of phenol can be so determined. Hydroquinone does not interfere if present in concentrations equal to phenol, but if the ratio is increased to 10 times that of phenol the absorbance increases about 13%. Diphenylphenylenediamine does not interfere since it is not extracted by the base. *p*-Hydroxydiphenylamine reacts with the reagent to produce a color which absorbs in the same region as the phenol color.

Phenols can be separated by paper chromatography after forming the azo compound with diazotized *p*-nitroaniline. The steam distillate from tobacco smoke so treated showed phenol, *o*-cresol, *m*-cresol, and guaiacol.[14]

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[10] R. M. Pearson, *Analyst* **80,** 656-64 (1955).

[11] Harold Hart, *Anal. Chem.* **24,** 1500-1 (1952).

[12] E. F. Mohler, Jr., and L. N. Jacobs, *Ibid.* **29,** 1369-74 (1957).

[13] R. J. Lacoste, S. H. Venable, and J. C. Stone, *Ibid.* **31,** 1246-9 (1959).

If aqueous phenolic samples are to be retained more than 6 hours before analysis add 1 gram of copper sulfate pentahydrate to avoid biological decomposition.[15] Addition of acetic acid to biological liquids has a similar effect.[16]

o-Cresol and phenol are determined in the presence of *p*-cresol and *m*-cresol by dissolving the sample in 4:1 sulfuric acid containing 50 grams per liter of oxalic acid, heating at 50° for 1 hour and at 100° for 5 minutes, and reading with a green filter followed with a blue filter. In these methods[17] 1,3,4-xylenol interferes with the determinations of *p*-cresol, *o*-cresol, and phenol, 1,2,4-xylenol interferes in the determination of *p*-cresol, and 1,3,5-xylenol with the determination of *m*-cresol.

Samples—*Air.*[18] Pass through 45 ml. of 0.5% sodium bicarbonate solution. By proper handling this will determine 2-10 parts per billion.

Polyoxyethylene phenyl ethers. The ether should have the following general formula: $[C_6H_5O(CH_2CH_2O)_xCH_2OH]$ where x equals from 1 to 5. The method may not be applicable when x is greater than 5.

Dissolve a 10-25 gram sample containing up to 1.4 mg. of phenols in 100 ml. of 0.4% sodium hydroxide solution. Shake for 1 minute with 100 ml. of chloroform saturated with water. Discard the chloroform layer and repeat with 100 ml. and 100 ml. of chloroform. The phenols remain in the aqueous alkaline layer. Filter the alkaline layer for direct reading in the ultraviolet.

Waste water. Adjust the pH of a 900-ml. sample to 12 with solid potassium hydroxide. Add 10 grams of sodium chloride and 10 ml. of carbon tetrachloride. Shake mechanically for 30 minutes to extract the oil and discard this extract. Filter if suspended solids are present to avoid subsequent emulsification difficulties. Adjust the pH to 5 with hydrochloric acid and add exactly 10 ml. of tributyl phosphate to the sample in a bottle to extract phenols. Shake mechanically for 30 minutes. Remove the phenols concentrate by adding water until the upper layer can be removed from the neck of the bottle with a pipet or hypodermic syringe. Read in the ultraviolet.

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[14] C. H. Rayburn, W. R. Harlan, and H. R. Hanmer, *Ibid.* **25,** 1419 (1953).

[15] G. Noisette, *Eau* **40,** 71-2 (1953).

[16] V. Bergerova-Fiseroua and S. Skramousky, *Pracovní Lékařství* **4,** 64-75 (1952).

[17] L. Nicolas and R. Burel, *Chim. anal.* **38,** 316-21 (1956).

[18] M. M. Braverman, S. Hochheiser, and M. B. Jacobs, *Am. Ind. Hyg. Assoc. Quart.* **18,** 132-4 (1957).

Hydrocarbons.[19] Extract a 100-200 gram sample three times with 10-ml. portions of 1% sodium hydroxide solution. Dilute the extract to 100 ml. with water. Cool below 10° and develop a 50-ml. aliquot with diazotized *p*-nitroaniline (Vol. III, p. 117).

Methyl methacrylate.[20] Cool an aqueous solution of the sample containing less than 6 mg. of phenols below 10°. Develop with diazotized *p*-nitroaniline (Vol. III, p. 117).

Procedure—*Direct reading in the ultraviolet.* The sample is in aqueous 0.4% sodium hydroxide solution which has been extracted with chloroform. Read at 288 mμ against 0.4% sodium hydroxide solution saturated with chloroform. If the transmittance is unduly low, dilute an aliquot with 0.4% sodium hydroxide saturated with chloroform before reading.

Difference reading in the ultraviolet. The sample consists of a concentrate in tributylphosphate. To prepare the quaternary hydroxide reagent, react stoichiometric amounts of pure trimethyloctadecylammonium chloride and potassium hydroxide in a minimum amount of absolute methanol. Chill and filter to remove precipitated potassium chloride. Dilute to about 0.1 *M* with methanol and store in a polyethylene bottle. The reagent will attack glass. Alternatively, pass a methanol solution of the halide such as tetra-*n*-butyl-ammonium iodide or tetraethylammonium bromide through a methanol-washed column of Amberlite IRA-400 resin and adjust the solution to about 0.1 *M* hydroxide concentration.

Mix 4 ml. of the phenolic extract with 1 ml. of quaternary hydroxide reagent. Mix 4 ml. of phenolic extract with 1 ml. of methanol as a reference solution. Read the developed sample against the reference solution. If the absorbance is above 0.8, dilute both solutions to bring the absorbance between 0.8 and 0.2. To perform the dilutions use a 4:1 mixture of tributyl phosphate and quaternary hydroxide reagent for the sample. Dilute the reference solution with a 4:1 mixture of tributyl phosphate and methanol.

Obtain an absorbance correction for cells and reagents by comparison of the 4:1 mixture of tributyl phosphate and quaternary hydroxide reagent with the 4:1 mixture of tributyl phosphate and methanol. Calculate the phenol concentration as follows:

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[19] E. Kh. Rud and S. Ya. Skochilova, *Zavodskaya Lab.* 22, 919 (1956).

[20] T. Takeuchi, M. Furusawa, and Y. Takayama, *Japan Analyst* 4, 568-71 (1955).

$$C = 12{,}500\ (A - A_c)\ F/Va$$

where C = phenol concentration in ppm.,
$A - A_c$ = the observed absorbance minus the correction for cells and reagents,
V = volume in ml. of the original water,
F = final dilution factor,
a = absorptivity of phenols in liters/gram-centimeter.

Separation and reading of phenol, cresols, xylenols, and ethylphenol.[21] The chromatographic column required is shown in Figure 4. A 500-mm. column with water as the stationary phase is sufficient for separation of phenols and cresols alone, but when xylenols or ethylphenols are present, a 900-mm. column is required with 30% methyl Cellosolve as the stationary phase.

For the 500-mm. column grind 56 grams of a 1:1 mixture of 100-mesh and 200-mesh silica gel in a porcelain mortar. Gradually add 42 ml. of water to it and grind to a powdery consistency. Add enough cyclohexane to form a mobile slurry and pack the column with a glass rod, drawing off excess cyclohexane. Add cyclohexane to the top of the column and run through under 15 lb. per square inch pressure. The rate of flow should be about 120 ml. per hour. When that is attained leave the cyclohexane about 1-2 cm. above the packing and the column is ready for use.

The packing of the 900-mm. column is similar in detail but requires 100 grams of silica gel and 76 ml. of 30% aqueous methyl Cellosolve.

Dissolve the sample containing not more than 2 mg. of each phenol component in cyclohexane and dilute to a known volume. Alternatively, if any component comes through at a concentration too high to read well, that fraction is diluted further. Draw down the cyclohexane in the column until the adsorbant is just covered followed with an aliquot of the sample solution. Apply pressure of 15 lb. per square inch and add additional cyclohexane as the liquid level reaches the top of the silica gel. Collect the eluate in 5-ml. fractions and continue washing with cyclohexane until all phenols are washed through the column. If phenol is present this will require about 450 ml. for the short column or 750 ml. for the long column. Read each fraction at 270 mμ and plot the optical densities against the volume of the percolate in ml. to locate the absorption peaks of the following 6 groups: (1) 2,6-xylenol; (2) *o*-ethylphenol; 2,3-; 2,4-; 2,5-xylenol;

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[21] R. M. Pearson, *Analyst* **80**, 656-64 (1955).

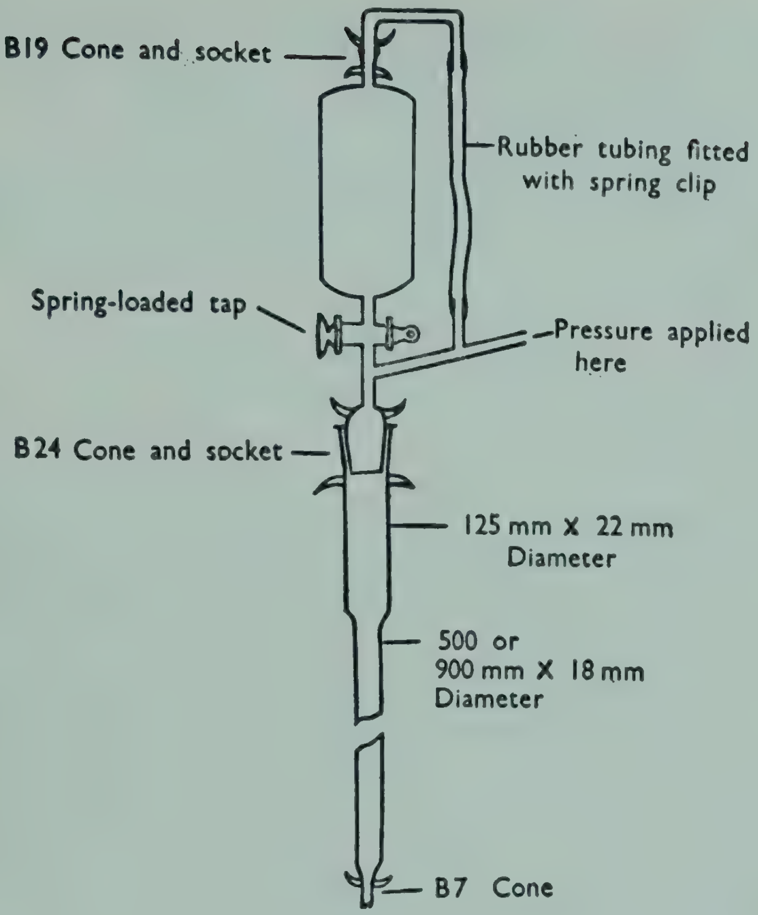
B19 Cone and socket
Rubber tubing fitted with spring clip
Spring-loaded tap
Pressure applied here
B24 Cone and socket
125 mm X 22 mm Diameter
500 or 900 mm X 18 mm Diameter
B7 Cone

FIG. 4

Chromatographic column and reservoir for separation of phenols

(3) *m*- and *p*-ethylphenols and 3,5-xylenol; (4) 3,4-xylenol and *o*-cresol; (5) *m*- and *p*-cresol; and (6) phenol. Calculate the concentration of each individual phenol in each fraction when not present in a mixture, for example, *o*-cresol if 3,4-xylenol is not present, as follows:

$$C_a = \frac{D \times V}{E_a}$$

where

C_a = concentration of the component a in the fraction
D = total optical density of the fraction
E_a = extinction coefficient of the component a
V = total volume of the fraction,

both D and E_a being measured at the same wave length.

To determine the percentage of a phenol that leaves the column as an individual peak, the optical densities of the fractions of percolate that make up the peak are measured at the appropriate wave length, multiplied by the volume of each fraction and totalled. The total optical density obtained is divided by the extinction coefficient of the particular phenol in question, thus giving the amount of the phenol, in mg. in the sample.

To determine a mixture of phenols leaving the column in one fraction calculate as follows:

$$D - E_1X_1 + E_2X_2 \ldots + E_nX_n$$

where X_n = the mole fraction of each component
D = total optical density
E_n = the extinction coefficient of each component.

Choose the absorption peaks of the components to be determined and then determine the extinction coefficient of each selected wave length. To determine the extinction coefficients, dilute a 0.1-gram sample to 100 ml. with cyclohexane, and read at the selected wave lengths. From the optical densities observed, calculate the optical density which would be obtained with a concentration of 1 mg. of the phenol per ml. of cyclohexane as the extinction coefficient. Measure the total optical density of the mixture at the wave lengths selected and substitute in the formula to form simultaneous equations.

This is best clarified by an example using 2-ethylphenol and the 3 xylenols which come through with it. Data for use are:

	Extinction coefficient at			
	272mμ	279mμ	282mμ	286mμ
2-Ethylphenol	16.45	15.25	6.55	1.43
2:3-Xylenol	11.30	12.88	6.88	8.50
2:4-Xylenol	12.35	16.90	15.20	15.85
2:5-Xylenol	14.48	13.78	14.98	4.73

The total optical densities of the four-component mixture at the four wave lengths are then measured. Substitution of these values and the values of the extinction coefficients gives four simultaneous equations as follows

$$D_1 = 16.45X_1 + 11.30X_2 + 12.35X_3 + 14.48X_4$$

$$D_2 = 15.25X_1 + 12.88X_2 + 16.90X_3 + 13.78X_4$$

$$D_3 = 6.55X_1 + 6.08X_2 + 15.20X_3 + 14.98X_4$$

$$D_4 = 1.43X_1 + 8.50X_2 + 15.85X_3 + 4.73X_4,$$

where X_1, X_2, X_3 and X_4 represents 2-ethylphenol, 2:3-, 2:4- and 2:5-xylenols, respectively, and D_1, D_2, D_3 and D_4 the total optical densities of the sample measured at 272mμ, 279mμ, 282mμ, and 286mμ, respectively.

From these equations the amount of each phenol present, in mg, may be expressed as follows

$$X_1 = +0.2715D_1 - 0.1873D_2 - 0.1215D_3 + 0.1042D_4$$
$$X_2 = -0.3202D_1 + 0.3485D_2 + 0.0369D_3 - 0.1549D_4$$
$$X_3 = -0.0134D_1 + 0.0190D_2 - 0.0283D_3 + 0.0805D_4$$
$$X_4 = +0.0234D_1 - 0.0745D_2 + 0.1325D_3 - 0.0652D_4.$$

Thus, once the extinction coefficients have been established, it is only necessary to measure the optical densities of an unknown mixture at the four selected wave lengths and substitute them in the above expressions in order to obtain the amount of each component, in mg, present in the weight of sample added to the chromatographic column.

By diazotized p-*nitroaniline.*[22] *Phenol,* o- *and* m-*cresols, and guaiacol in cigarette smoke.* Smoke 10 cigarettes of uniform weight according to a technic of smoking and smoke collection in which puffs of 35-ml. volume and 2-seconds duration are taken at intervals of 1 minute until 47 mm. of the cigarette is consumed. To obtain the total volume of smoke, multiply the number of puffs by 35. Let the smoke settle for 20 minutes and wash into a collection flask alternatively with hot water and 95% ethanol. Steam-distil at the pH of the main smoke stream and collect 250 ml. of distillate.

Extract the distillate with 100 ml. of ether followed by two 50-ml. portions of ether. Extract the combined ether extracts with three 20-ml. portions of 5% sodium hydroxide solution. Acidify the alkaline extract with 1:4 sulfuric acid and steam distil, collecting 250 ml. Develop with the diazo reagent.

To prepare the reagent, dissolve 0.7 grams of *p*-nitroaniline in 9 ml. of concentrated hydrochloric acid and dilute to 100 ml. Cool 4 ml. in ice and add 5 ml. of chilled 1% sodium nitrite solution. Dilute to 100 ml. with ice water. Store in an ice bath. Cool nine 25-ml. aliquots at 0° and add diazo reagent ranging in equal increments from 1.6-4.0 ml. Add 5 ml. of saturated sodium bicarbonate solution to each and let stand for 15 minutes in the ice bath. Acidify with 1 ml. of 1:4 sulfuric acid and extract with 3 portions of ether. Combine the ether extracts of each solution and evaporate each to dryness. Take up each in 1 ml. of acetone.

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[22] C. H. Rayburn, W. R. Harlan, and H. R. Hanmer, *Anal. Chem.* 25, 1419 (1953) (1 page).

Saturate 46.5 × 57 cm. sheets of filter paper by spraying with 1% sodium carbonate solution and air dry. Apply a 0.5-ml aliquot of the phenol in acetone as a band 40 cm. × 0.5 cm. at a 5 cm. distance from the bottom edge of the paper. Condition the paper for 1 hour and develop 15-16 hours with a solvent mixture containing 100 ml. of ether, 100 ml. of 30-65° petroleum ether, and 40 ml. of 50% methanol in a cylindrical glass jar. Perfect air tightness is not necessary as slight evaporation improves separation while excessive evaporation gives bands which are too close together. Identify the phenol, guaiacol, *m*-cresol, and *o*-cresol by color and position as follows:

1. a violet band about 5-8 cm. above the starting line is guaiacol;
2. a broad rose band about 15-20 cm. above the starting band is phenol;
3. a narrower red band above the rose band is *m*-cresol;
4. a reddish-purple band above the *m*-cresol band is *o*-cresol.

Bands appearing above these derivatives contain mixtures of other phenolic derivatives in small amounts. Cut the paper lengthwise with each derivative on a separate strip. Elute with a downward development with 80% isopropanol solution and allow the solvent to drip into a beaker. After elution is complete allow the solvent to evaporate. Dissolve the residues in a small amount of 20% sodium carbonate solution in isopropanol and filter through cotton. Dilute the guaiacol and cresol derivatives to 25 ml. and the phenol derivative to 100 ml. Read at 510 mμ for guaiacol and cresol and at 490 mμ for phenol. Plot the absorbance of each phenol against the volume of diazo reagent used and average the absorbances near the maximum for each curve in order to obtain the maximum for the compound.

Prepare a 250-ml. solution containing 2 mg. of phenol and 0.3 mg. each of guaiacol, *m*-cresol, and *o*-cresol. Using amounts of reagent varying from 0.7-1.8 ml., separate the phenols into 25-ml. aliquots, and follow the procedure as if these aliquots were the steam distillate. Calculate the concentration of each phenol in the smoke from the average maximum found for the known phenols as follows:

$$\frac{\text{absorbance of unknown}}{\text{absorbance of known}} \times \frac{\text{mg. known in 250-ml. solution}}{\text{volume of smoke in liters}} = \text{mg. per liter of smoke.}$$

By ferrous ion. Phenolic acids and catecholic phenols in urine.[23] Mix 1 ml. of sample with 1 ml. of 3:1 hydrochloric acid in an open tube at 100° for 1 hour. Cool and extract with 5, 3, and 3 ml. of ether. Evaporate the combined ether extracts at room temperature in a stream of air. Take up the residue in 0.5 ml. of water. Add 0.5 ml. of reagent containing 0.1% of ferrous sulfate heptahydrate and 0.5% of sodium potassium tartrate. Add 10 ml. of 10% ammonium acetate solution and 1 ml. of 1:6.5 ammonium hydroxide. Read after 20 minutes at 560 mμ against a reagent blank. An appropriate standard contains 0.25 mg. of resorcinol.

By m-*nitroaniline.*[24] *In salicylic acid.* Dissolve a 1-gram sample in 50 ml. of 10% sodium acetate solution and add 50 ml. of water. Mix and take a 1-ml. aliquot.

Add 10 ml. of buffer solution containing 5.3% of sodium dihydrogen phosphate dihydrate and 2.38% of disodium hydrogen phosphate dodecahydrate to adjust the pH to 5.5-6. Add 1 ml. of diazotized *m*-nitroaniline reagent (page 89) and let stand 15 minutes to allow the phenol to couple with the dye.

Add 1:5 hydrochloric acid until strongly acidic and shake twice with 30-ml. portions of benzene. Shake the benzene solution 3-4 times with a saturated sodium bicarbonate solution to remove dissolved dye. Shake twice with 40-ml. portions of 1% sodium hydroxide solution to extract the dye of the phenol from the benzene. Filter, wash, and dilute to 50 ml. Read immediately at 424 mμ.

By 4-aminoantipyrine. 1,3-Butanediol dimethacrylate. Shake a 5-ml. sample for 1 minute with 5 ml. of 4% sodium hydroxide solution. If the concentration of phenol is high, dilute the basic aqueous layer with 4% sodium hydroxide solution.

To 2 ml. of alkaline sample solution, add 18 ml. of a buffer containing 2.47% of boric acid and 0.4% of sodium hydroxide. Add 0.1 ml. of 3% 4-aminoantipyrine solution and 0.2 ml. of 10% potassium ferricyanide solution. The pH should be 9.1-9.2. Read at 510 mμ.

By p-*aminodimethylaniline.* As reagent add 20 grams of *p*-aminodimethylaniline to 100 ml. of 5:3 sulfuric acid. When dissolved dilute with water to 100 ml. For use dilute 1:19 with water.

Dilute the solution of sample in sodium bicarbonate solution to 125 ml. with water. Add 4 drops of reagent and mix. Add 0.1% calcium hypochlorite solution dropwise until the initial yellow color becomes blue or colorless. Extract the color with an appropriate volume of chloroform and filter the extract. Read at 600 mμ after 30 minutes.

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[23] S. L. Tompsett, *J. Pharm. and Pharmacol.* 10, 157-61 (1958).

[24] E. V. Schivizhoffen, *Z. anal. Chem.* 145, 184-7 (1954).

METHOXYPHENOL, GUAIACOL

In the presence of alkaline ferricyanide, catechol forms an acidic dye with 4-aminoantipyrine and exists as an anion, while guaiacol forms a neutral dye with 4-aminoantipyrine, has no ionizable hydrogen, and is therefore soluble in chloroform. This permits the determination of guaiacol in the presence of large amounts of catechol.[25]

Since the concentration of catechol should not exceed 0.028 mg. per ml., it is necessary to determine the concentration of catechol in the sample. A pH of 10.45-10.55 is chosen because a lower pH gives higher ratios of blank to net readings and a higher pH lowers the sensitivity of the test. Sodium carbonate gives a lower reagent blank and greater consistency than ammonium hydroxide as the base.

The extraction is carried out in a beaker rather than a separatory funnel to avoid contamination with stopcock grease which imparts color to the chloroform extract. Beer's law is followed in the presence of 0.1 mg. of catechol per ml. for up to 0.6 ppm. of guaiacol. When the concentrations of guaiacol are higher than 0.6 ppm., more chloroform is used to bring the absorbance within the instrumental range.

Guaiacol in cigarette smoke is converted to the azo compound of *p*-nitroaniline. Thereafter it is separated from phenol and cresols by paper chromatography (page 90).

Procedure—Adjust the catechol concentration of a 250-ml. sample containing up to 0.6 ppm. of guaiacol to be the same as that used in preparation of the standard curve. Add 21.2% sodium carbonate solution to bring the pH to 10.45-10.55. Add 2 ml. of 0.75% 4-aminoantipyrine solution, and stir. Add 5 ml. of 2% potassium ferricyanide solution and stir vigorously for a few seconds. Immediately add 12 ml. of chloroform, stir for 5 minutes, and allow the chloroform to settle in 30 seconds. Pipet off the chloroform layer and read at 460 mμ.

o-PHENYLPHENOL

A color complex is formed between *o*-phenylphenol and titanium sulfate with a 1:1 molar ratio having a maximum density at 450 mμ.[26] Since

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[25] D. N. Rosenblatt, M. M. Demek, and J. Epstein, *Anal. Chem.* **26**, 1655-6 (1954).

[26] P. H. Caulfield and R. J. Robinson, *Ibid.* **25**, 982-3 (1953); Cf. Vol. III, pp. 123-4.

o-phenylphenol sublimes upon heating, it is necessary to react the solid *o*-phenylphenol with the titanium reagent immediately after evaporation of the solvent. Full color is developed in 5 minutes and is stable for at least 2 hours. It is not effected by sunlight. Beer's law is followed for 0.004-0.16 mg. of *o*-phenylphenol per ml. of titanium reagent.

The following phenols form colored complexes with the reagent with maximum absorbance at the wave length indicated: *m*-aminophenol 417 mμ, *p*-aminophenol 418 mμ, *p*-bromophenol 473 mμ, catechol 460 mμ, *p*-chlorophenol 465 mμ, *o*-cresol 455 mμ, 2,4-dichlorophenol 490 mμ, *p*-ethylphenol 473 mμ, hydroquinone 503 mμ, *p*-hydroxybenzaldehyde 420 mμ, *p*-hydroxybenzoic acid 390 mμ, 5-hydroxy-1,3-dimethylphenol 453 mμ, 1-naphthol 440 mμ and 655 mμ, 2-naphthol 435 mμ, *p*-nitrophenol 451 mμ, phenol 455 mμ, *p*-phenylphenol 482 mμ, phenyl salicylate 435 mμ, phloroglucinol 415 mμ, pyrogallol 460 mμ, resorcinol 405 mμ, *p*-*tert*-butylphenol 469 mμ, and thymol 438 mμ. *o*-Aminophenol and *o*-nitrophenol do not exhibit any visible spectrum with the reagent. Tribromophenol will not dissolve in the reagent.

o-Phenylphenol gives a cherry red color with Brentamine fast red GG with an absorption maximum at 494 mμ.[27] The reaction is carried out in strong sodium acetate solution and developed with sodium carbonate. The color is stable for 24 hours in the presence of gum acacia. For this purpose the sample should be in 4% sodium hydroxide solution. Interferences can be minimized by solution of the sample in cyclohexane and extraction of the *o*-phenylphenol with the sodium hydroxide solution. Various colors are given with this reagent by other phenols not substituted in the *para* position. In the absence of interferences it will detect 0.001 mg. per ml. Accuracy is to ±5% except for timber samples where it is somewhat less accurate.

o-Phenylphenol is also read directly in the ultraviolet, although this method is subject to more interferences than the colorimetric methods. The peak at 284 mμ in cyclohexane is preferable to that at 250 mμ as subject to less interference. In some cases interferences which remain after sodium hydroxide extraction are overcome by use of the hypsochromic effect, a shift of the absorption maxima to a shorter wave length which occurs when the solution is acidified and the phenoxide changes to free phenol. This requires that the absorption spectra of the interfering substance does not change when acidified. The lower limit of detection is about 0.001-0.002 mg. per ml.

The strong blue-violet fluorescence in ethanolic sodium hydroxide is also readable. The concentration of alkali present is not important. The only known interferences are the green of *o-tert*-butylphenol and the green of *o-iso*propylphenol. It will detect 0.0005 mg. per ml. but is only accurate to ±20%.

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[27] D. Harvey and G. E. Penketh, *Analyst* **82**, 498-503 (1957).

Samples—*Sodium chloride with added fungicide.* Dissolve a 1-gram sample in 50 ml. of water. Add 10 ml. of 4% sodium hydroxide solution to ensure complete solution of the *o*-phenylphenol and dilute to 100 ml. with water. Read in the ultraviolet or develop with Brentamine fast red GG.

Disinfectant formulations. Dissolve a 5-gram sample in cyclohexane, if soluble, and dilute to 100 ml. If the sample is aqueous, acidify and extract with five 10-ml. portions of cyclohexane. Read in the ultraviolet. If interferences are found to be present, extract the *o*-phenylphenol from the cyclohexane with five 10-ml. portions of 2% sodium hydroxide solution.

Citrus fruits. Roughly mince one whole fruit and add 100 ml. of water and 2 ml. of glacial acetic acid. Steam distil the sample until the distillate is free from *o*-phenylphenol. A distillate of less than 500 ml. is usually sufficient, but a further 500 ml. should be collected and read separately. Dilute the first distillate to 500 ml. with water. To a 100-ml. aliquot, add 5 ml. of 1:10 hydrochloric acid and extract the *o*-phenylphenol with five 10-ml. portions of cyclohexane. Extract the combined cyclohexane layers with five 10-ml. portions of 2% sodium hydroxide solution to separate the *o*-phenylphenol from oils. Develop in the ultraviolet or with Brentamine fast red GG.

Alternatively, roughly mince one whole fruit, add 250 ml. of cyclohexane containing 1% of glacial acetic acid to ensure extractibility of the *o*-phenylphenol, and macerate for 5 minutes in high-speed mechanical equipment. Allow to settle and extract 50 ml. of the cyclohexane layer with seven 5-ml. portions of 3% sodium hydroxide solution. If emulsification interferes, add 15% sodium sulfate decahydrate solution to the sodium hydroxide. Dilute the combined extracts to 50 ml. with 3% sodium hydroxide solution and develop in the ultraviolet or with Brentamine fast red GG. If the sample has been treated with an alkali soluble dye, use the colorimetric method with Brentamine fast red GG with an appropriate correction.

Timber. Add 100 ml. of water to a 1-5 gram sample of drillings or shavings. Add 2 ml. of glacial acetic acid and steam distil until the distillate is free from *o*-phenylphenol. For samples containing up to 1% of *o*-phenylphenol, 500 ml. of distillate is sufficient, but a further 500 ml. should be collected and developed separately to ensure that all *o*-phenylphenol has been liberated. Develop with Brentamine fast red GG.

Paper and canvas. To a 1-gram shredded sample, add 100 ml. of 0.4% sodium hydroxide solution and reflux for 1 hour. Cool, filter, and read in the ultraviolet or develop with Brentamine fast red GG.

Procedure—*By titanium sulfate.* Digest 0.45 gram of anhydrous titanium dioxide in 1 liter of concentrated sulfuric acid for several hours at 300° until a clear solution results. Separate *o*-phenylphenol from interferences if necessary by steam distillation, by leaching with acetone or diethyl ether, or by extraction from an acidified aqueous solution with ether. Evaporate any solvent and immediately react a 0.5-4 mg. sample with 25 ml. of reagent. Stir for 5 minutes to dissolve and fully develop the color. Read at 450 mμ against concentrated sulfuric acid containing the same concentration of phenol as the sample.

Brentamine fast red GG. Neutralize an aliquot of the aqueous alkaline sample to pH 7-9 and dilute so that 1 ml. contains 0.001-0.01 mg. of *o*-phenylphenol. To a 10-ml. aliquot, add 1 ml. of 1% gum acacia solution, 1 ml. of 50% sodium acetate solution, and 1 ml. of 0.5% Brentamine fast red GG solution. After 1 minute, add 2 ml. of 20% sodium carbonate solution and read at 494 mμ against a reagent blank.

In the ultraviolet. Cyclohexane solutions. Read an aliquot of the sample containing 0.005-0.025 mg. of *o*-phenylphenol per ml. at 284 and 295 mμ. If the results at the two wave lengths, when translated from a calibration curve, do not agree, interference is indicated.

Sodium hydroxide solutions. Follow the procedure for cyclohexane solutions, using the wave lengths of 300 and 320 mμ.

Sodium hydroxide solutions containing interferences. Dilute a 20-ml. aliquot containing 0.005-0.025 mg. of *o*-phenylphenol per ml. to 50 ml. with water and read at 285 and 315 mμ against a blank containing sodium hydroxide of the same concentration as the sample. To a similar 20-ml. aliquot, add 5 ml. of concentrated hydrochloric acid and dilute to 50 ml. with ethanol to retain the phenol in solution. Read at 285 and 315 mμ against a reagent blank. To obtain the difference optical densities, subtract the two sets of measurements and compare to difference optical densities obtained with standard solutions of 5 mg. of *o*-phenylphenol in 100-ml. sodium hydroxide solution similarly treated.

By fluorescence. Dilute an aliquot of not over 10 ml. of sample containing 0.01-0.1 mg. of *o*-phenylphenol in aqueous alkaline solution to 100 ml. with 95% ethanol. Compare the fluorescence with that of standards in similar tubes. The standards must contain the same volume and concentration of alkali as the aliquot of sample used.

Wingstay S, an Alkylated Phenol, Ionol, 2,6-Di-*tert*-butyl-4-methylphenol, and Antioxidant 2246, Methylenebis-2,2′ (6-tertbutyl-4-methylphenol)

These three proprietaries are used as rubber antioxidants. The first is an unidentified alkylphenol, the other two substituted cresols. Since they undergo a change in the ultraviolet spectrum as a function of basicity, they can be determined by measuring in neutral solution where the antioxidant has a low absorptivity and in potassium hydroxide solution where there is an increase in absorbance.[28] Thus error due to background absorption is corrected. Beer's law is followed for 0.005-0.05 gram per liter.

Sample—*GR-S rubber.* Sheet the sample to a thickness of 0.02 inch and cut into 1 × 5 cm. strips. Add a 6-gram sample to 100 ml. of absolute ethanol, 1 strip at a time to prevent sticking. Reflux for 60 minutes. Pour off the solvent and rinse the sample with three 10-ml. portions of absolute ethanol. Dilute the combined extracts and washings to 250 ml. with absolute ethanol.

Procedure—Take two 5-ml. aliquots of the sample solution. Add 5 ml. of 5.6% alcoholic potassium hydroxide solution to one. Dilute each to 50 ml. with absolute ethanol. Prepare a blank by diluting 5 ml. of 5.6% alcoholic potassium hydroxide solution to 50 ml. with absolute ethanol. Read the neutral extract at 301 mμ against absolute ethanol and read the alkaline aliquot against the potassium hydroxide blank. Calculate alkylated phenol as follows:

$$\%\ \text{alkylated phenol} = \frac{(A_{\text{base}} - A_{\text{neutral}}) \times V \times E \times 0.1}{S \times a \times D \times b}$$

where

a = absorptivity, liter gram^{-1} centimeter^{-1}

A_{base} = absorbance in basic solution

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[28] Coe W. Wadelin, *Anal. Chem.* 28, 1530-1 (1956).

A_{neutral} = absorbance in neutral solution
c = concentration in grams/liter
b = cell length, centimeters
S = sample weight in grams
V = volume of combined extracts, milliliters
D = volume of aliquot, milliliters
E = volume to which aliquot is diluted

$$a = \frac{(A_{\text{base}} - A_{\text{neutral}})}{c \times b}.$$

MISCELLANEOUS PHENOLIC RUBBER ANTIOXIDANTS

Other phenolic antioxidants including those shown in the previous topic are determined in ethanol or methanol by coupling with diazotized *p*-nitroaniline.[29] The determinable compounds are shown in Table 8. The method is applicable to raw or cured natural or synthetic polymers. Compounding ingredients do not interfere if not phenolic. If oil is present as an extender add chloroform to the solution in sufficient amount to remove turbidity. Highly oxidized samples give low results as the oxidized antioxidant reacts differently or not at all. Mixtures may be determinable by simultaneous equations if sufficiently far apart in absorption maxima.

Procedure—*Rubber.* Sheet thinly or powder on a Wiley mill. Wrap a 2-gram sample in pre-extracted sizing cloth. Extract for 16 hours with 95% ethanol or methanol. Dilute the extract to 100 ml. with 95% ethanol.

As a coupling reagent dissolve 2.8 grams of *p*-nitroaniline in 32 ml. of hot concentrated hydrochloric acid. Dilute to 250 ml. with water at room temperature. Dissolve 1.44 grams of sodium nitrite in water and dilute to 250 ml. at room temperature. Chill 25 ml. of each to room temperature and mix well. Let warm to room temperature and add 1 ml. of 1% urea solution to destroy excess nitrite. Although this clouds after an hour or so it is useable for several hours.

Mix 10 ml. of the diluted sample in 95% ethanol with 2 ml. of coupling reagent. Mix and add 3 ml. of 16% sodium hydroxide solution. Dilute to 100 ml. with 95% ethanol or methanol. Read at the appropriate wave length according to the compound as shown in Table 8. Since these are variable commercial compounds for maximum accuracy the standard curve must have been prepared from the same batch. The blank is a di-

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[29] C. L. Hilton, *Ibid.* 32, 383-7 (1960).

TABLE 8. COMPOSITION AND WAVE LENGTH OF MAXIMUM ABSORPTIVITY FOR PHENOLIC ANTIOXIDANTS

Antioxidant	*Composition*	*Wave length Max.*, Mμ
AgeRite Alba[a]	Hydroquinone monobenzyl ether	565
AgeRite Spar[a]	Styrenated phenol	548
AgeRite Superlite[a]	A polyalkyl polyphenol	560
Antioxidant 5[a]	Not disclosed	585
Antioxidant 425[a]	2,2′-Methylenebis(6-*tert*-butyl-4-methylphenol)	585
Antioxidant 2246[a]	2,2′-Methylenebis(6-*tert*-butyl-4-methylphenol)	578
Deenax[a]	2,6-Di-*tert*-butyl-*p*-cresol	Does not couple
Ionol[a]	2,6-Di-*tert*-butyl-*p*-cresol	Does not couple
1-Naphthol	1-Naphthol	598
2-Naphthol	2-Naphthol	540
Naugawhite[a]	Alkylated phenol	580
Nevastain A[a]	Not disclosed	550
Nevastain B[a]	Not disclosed	550
Nonyl phenol	Nonyl phenol	538
p-Phenyl phenol	*p*-Phenyl phenol	548
Polygard[a]	Tris(nonylated phenyl) phosphite	Must be hydrolyzed before it will couple
Santovar A[a]	2,5-Di-*tert*-amylhydroquinone	Color too weak
Santovar O[a]	2,5-Di-*tert*-butylhydroquinone	Color too weak
Santowhite Crystals[a]	4,4′-Thiobis(6-*tert*-butyl-2-methylphenol)	565
Santowhite MK[a]	Reaction product of 6-*tert*-butyl-*m*-cresol and SCl_2	560
Santowhite Powder[a]	4,4′-Butylidene-bis(3-methyl-6-*tert*-butylphenol)	Color too weak
Solux[a]	*N*-*p*-Hydroxyphenylmorpholine	Color too weak
Stabilite White Powder[a]	Not disclosed	Color too weak
Styphen I[a]	Styrenated phenol	558
Wingstay S[a]	Styrenated phenol	545
Wingstay T[a]	A hindered phenol	590

[a] Trademark.

luted sample equivalent to the developed one but with the coupling agent omitted.

o-HYDROXYPHENYL ACETIC ACID

As little as 0.003 mg. of *o*-hydroxyphenylacetic acid in penicillin can be determined by the yellow color developed by a modification of the nitrosation method for phenols.[30] Other methods for phenols cannot be applied to the determination of the acid in penicillin fermentations since it is difficult to distinguish the acid from nonphenolic interfering substances in the medium. By this method the color of the interferences is too low to affect the results.

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[30] S. C. Pan, *Ibid.* **27**, 65-7 (1955); Cf. Vol. III, p. 125.

Amyl acetate is used as a solvent to extract *o*-hyroxyphenylacetic acid from other phenolic substances in the fermentation medium. Only one extraction is employed and the percentage of *o*-hydroxyphenylacetic acid extracted, based on an extraction performed with pure *o*-hydroxyphenylacetic acid, is applied as a correction factor. Within the range of 0.05-0.5 mg. per ml. the extraction varies between 84 and 89%, 86% being taken as the correction factor. This percentage could be increased to 90% or higher by saturating the aqueous phase with anhydrous sodium sulfate or by using a larger volume of amyl acetate. This extraction procedure is specific for interferences in fermentation of cornsteep medium by penicillin-producing fungi and may not necessarily apply to other samples such as blood or urine.

The color is stable. It increases in intensity with an increase in sulfuric acid concentration, reaching a plateau at 1 *N*. Phenylacetic acid and penicillin-G do not interfere. Beer's law is followed up to 0.3 mg per 7 ml. of the reaction mixture. At concentrations over this point there is a deviation from a straight line, but the error introduced amounts to less than 5% up to 0.04 mg. of acid.

Procedure—*Cornsteep medium of penicillin-producing fungi.* Shake a 1-ml. cell-free filtrate with 1 ml. of 1:35 sulfuric acid and 5 ml. of amyl acetate for 30 seconds using the thumb as a stopper. Cork or rubber stoppers should be avoided. Centrifuge for 5 minutes to separate the layers. To an aliquot of the amyl acetate layer containing 0.003-0.03 mg. of *o*-hydroxyphenylacetic acid, add 0.1 ml. of 0.4% sodium hydroxide solution and evaporate to dryness at 50-70° under a gentle current of air. Dissolve in 1 ml. of water and add 1 ml. of 1:16 sulfuric acid and 0.1 ml. of 4% sodium nitrite solution. Cork loosely and heat at 100° for 10 minutes. Cool, add 5 ml. of 1:3 ammonium hydroxide to neutralize, and read against a reagent blank at 420 mμ. To correct to the amount present divide the result by 0.86.

2,4,6-TRICHLOROPHENOL

The use of 2,4,6-trichlorophenol for preservation of moist feedstuffs has led to its determination by 4-aminoantipyrine at pH 7.6-8.[31] At that buffered pH the color conforms to Beer's law up to 0.6 mg. per 100 ml. of final solution.

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[31] Roy U. Schenk, *Chemist Analyst* **49,** 14-5 (1960).

Procedure—Prepare the aqueous solution or dispersion. If interfering alkaline, neutral, or weakly acid materials such as phenol are present make the sample alkaline with sodium carbonate and steam-distil. Thereafter make the sample acid with phosphoric acid and steam-distil the 2,4,6-trichlorophenol.

Dilute a sample of steam-distillate containing up to 0.4 mg. of 2,4,6-trichlorophenol to 70 ml. Add 25 ml. of buffer prepared from 1.36% monopotassium phosphate solution adjusted to pH 7.6-8 by addition of 4% sodium hydroxide solution before final dilution. Add 0.5 ml. of 1% 4-aminoantipyrine solution and water to make up to about 95 ml. Add 0.25 ml of 8% potassium ferricyanide solution and dilute to 100 ml. Keep protected from light and read within 5-30 minutes at 500-525 mμ.

CRESOLS

Cresols are separable from phenol, xylenols, and ethylphenol chromatographically (page 86). Mixtures of *m*-cresol, *o*-cresol, and *p*-cresol (Cf. Vol. III, pp. 120-2) are analyzed for any two or all three cresols simultaneously by reading the absorbancies directly in isooctane in the ultraviolet.[32] Substances other than cresols, such as phenols and alkylphenols, which absorb between 271.5 mμ and 286.0 mμ interfere. Beer's law is followed when the concentrations showed absorbances between 0.5 and 0.9 at the characteristic wave length of the isomer. Slit widths should be chosen to provide spectral band widths of 5 A to increase the precision of the method.

Procedure—Dissolve a 0.05-gram sample in isooctane and dilute to a concentration of approximately 0.05 gram per liter of solution. Read at 272.8 mμ, 277.4 mμ, and 285.8 mμ for *m*-cresol, *o*-cresol, and *p*-cresol respectively. Calculate the concentration of each isomer as follows:

$$
\begin{aligned}
A_{272.8\,\mathrm{m}\mu} &= 16.41\,c_o + 15.70\,c_m + 14.68\,c_p \\
A_{277.4\,\mathrm{m}\mu} &= 15.89\,c_o + 10.28\,c_m + 15.33\,c_p \\
A_{285.8\,\mathrm{m}\mu} &= 0.716\,c_o + 0.905\,c_m + 19.30\,c_p
\end{aligned}
$$

or

$$
\begin{aligned}
c_o &= -0.122A_{272.8} - 0.1918A_{277.4} - 0.05945A_{285.8} \\
c_m &= 0.1958A_{272.8} - 0.2027A_{277.4} + 0.01219A_{285.8} \\
c_p &= -0.00465A_{272.8} + 0.00239A_{277.4} + 0.05345A_{285.8}
\end{aligned}
$$

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[32] G. E. Carney and J. K. Sanford, *Anal. Chem.* 25, 1417-8 (1953).

where c = the concentration of a constituent in liters per gram liter of solution.

p-CRESOL

Diazotized hydrazinobenzene sulfonic acid is a reagent for *p*-cresol in the range of 0.01-0.14 mg.[33] 2,6-Dibromoquinone chloroimide, which does not react with para substituted phenols, couples with interfering phenols to produce indophenols which are separated from *p*-cresol by steam distilation. The final color is stable for at least 4 hours and is not effected by light.

The reaction of *p*-cresol with α-nitroso-β-naphthol in acetic acid followed by dilution with water and diazotizing determines *p*-cresol.[34] So long as phenols contain no substituent in the *para* position they do not interfere. A related method is to treat the sample in 1:1 acetic acid with sodium nitrite and hydrochloric acid and β-nitroso-α-naphthol. The excess 2-nitroso-1-naphthol is extracted with benzene before reading the resulting red color.

Sample—*Waste water.* Determine the total phenol content of the sample by the antipyrine method (Vol. III, p. 119) or the 2,6-dibromoquinone chloroimide method (Vol. III, p. 118) to obtain a phenol value to provide for adding an appropriate excess of chloroimide reagent.

To a 250-ml. or larger sample add 4 drops of methyl orange and 1:35 sulfuric acid until the sample is definitely acidic. Shake with 40 ml. of ethyl ether followed with four 25-ml. portions of ether. Shake the combined ether layers three times with one 4-ml. portion of 10% sodium hydroxide solution and two 3-ml. portions of the sodium hydroxide solution. Heat the combined alkaline extracts to remove ether. Add 2 grams of anhydrous sodium carbonate to prevent decomposition of 2,6-dibromoquinone chloroimide and dilute to 30 ml. with water. Adjust the pH to 8 with dilute sulfuric acid and dilute to 50 ml. Use the solution containing less than 4 mg. of phenols, as previously determined, with the exception of *p*-cresol, or a suitable aliquot diluted to 50 ml. Add 5 ml. of 1% 2,6-dibromoquinone chloroimide solution in 95% ethanol. Allow the blue color of the indophenols to develop overnight. Cautiously add with stirring 1 gram of ascorbic acid. The indophenols turn red. Dilute to 100 ml. and steam distil a 10-ml. aliquot. Collect 30 ml. of the water-white steam distillate containing *p*-cresol. Develop with diazotized *p*-hydrazinobenzenesulfonic acid.

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[33] G. R. Tallon and R. D. Hepner, *Ibid.* 30, 1521-4 (1958).

[34] Jadwiga Mlodecka, *Chem. Anal.* (*Warsaw*) 4, 45-52 (1959).

Procedure—*By diazotized* p-*hydrazinobenzene sulfonic acid.* Dissolve 1 gram of *p*-hydrazinobenzenesulfonic acid and 0.3 gram of anhydrous sodium carbonate in 80 ml. of water by warming. Add 9 ml. of concentrated hydrochloric acid and dilute to 100 ml. Warm to redissolve any crystals which separate. Prepare the final reagent about 10 minutes before use by cooling 4 ml. of the *p*-hydrazinobenzene sulfonic acid solution to 10° and adding 5 ml. of chilled 1% sodium nitrite solution. Mix, maintain at 10° for 3-5 minutes, and dilute to 100 ml. with water at 10°. Dilute a portion of sample, free from phenol or *o*- or *m*-cresols and containing up to 0.1 mg. of *p*-cresol, to 40 ml. with water. Add 5 ml. of reagent and mix. Add 1 ml. of 1:7 ammonium hydroxide, mix, and dilute to 50 ml. with water. Read after 5 minutes at 495 mμ against a reagent blank.

By diazotized α-nitroso-β-naphthol. Mix 1 ml. of 1% α-nitroso-β-naphthol in glacial acetic acid with the sample containing 0.02-0.5 mg. of *p*-cresol in glacial acetic acid. Mix well, chill, and add to 25 ml. of chilled 1:10 hydrochloric acid. At once add 1 ml. of chilled 3.5% sodium nitrite solution. After 20 minutes dilute to 50 ml. with water. Filter and read at 500 mμ.

3,5-DINITRO-*o*-CRESOL

The sodium salt of 3,5-dinitro-*o*-cresol appears yellow in alkaline ethyl methyl ketone and appears colorless when acidified to the free acid. β-Carotene at levels of 0.01-0.03 mg. per gram of sample shows the same optical density in acid or alkaline solution. Based on these considerations 3,5-dinitro-*o*-cresol is determined in the presence of β-carotene in blood samples. In other samples, β-carotene is separated from 3,5-dinitro-*o*-cresol in chloroform solution with sodium carbonate solution.[35]

Sample—*Cow's blood.* Shake a 1-ml. sample containing 0.005-0.05 mg. of 3,5-dinitro-*o*-cresol per ml. with 5 ml. of ethyl methyl ketone. Add 1-2 grams of a 9:1 mixture of sodium chloride and sodium carbonate and shake vigorously for 30 seconds. Filter off the supernatant ethyl methyl ketone and read at 430 mμ against a ketone blank. Add 1 drop of concentrated hydrochloric acid to the extract and mix. If slight clouding occurs, centrifuge. Again measure at 430 mμ. Subtract the second reading from the first.

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[35] M. L. Fenwick and V. H. Parker, *Analyst* **80**, 774-6 (1955).

Locusts. Cut the locust into 3-4 pieces over the glass vessel of a homogenizer and add 25 ml. of chloroform. Add 25 ml. of 10% trichloroacetic acid solution and homogenize for 1 minute. Filter through four layers of gauze or cheese cloth and allow the chloroform to settle to the bottom. Pipet out 5 ml. of the chloroform layer, avoiding inclusion of any aqueous phase, and add 10 ml. of 2% sodium carbonate solution to the chloroform layer to extract the 3,5-dinitro-*o*-cresol from the β-carotene. Holding the thumb over the tube, shake vigorously for 10 seconds and let stand for 10 minutes. A cloudy upper layer does not effect the results. To 6 ml. of the sodium carbonate layer, add 5 ml. of ethyl methyl ketone and 1 gram of sodium chloride. Shake for a few seconds to salt out 3,5-dinitro-*o*-cresol and remove the ketone layer. Read the ketone layer at 430 mμ.

2,6-DI-*tert*-BUTYL-4-METHYLPHENOL

An important antioxidant for fats, rubber, petroleum products, plastics, and other organic substances, 2,6-di-*tert*-butyl-4-methylphenol is separated from interference by chromatograph on silica and read at 284 mμ.[36] Although the ultraviolet absorption spectrum of the antioxidant shows two maximum bands of approximately equal intensity at 276 and 284 mμ, the 284 mμ wave length is less subject to interferences.

For proper separation from other preservatives and substances containing phenolic hydroxyl groups in benzenoid or fused ring structures, including butylated hydroxyanisole, nordihydroguaiaretic acid, propyl gallate, citric acid, monoisopropyl citrate, and phosphoric acid, careful adjustment of the water in the silica gel to 13% is essential. Cyclohexane purified through the column can be used. An appropriate range for determination is 20-200 ppm.

Procedure—*Lard.* The apparatus is shown in Figures 5 and 6. Set up a chromatographic column 51 mm. in outside diameter and 42 inches long with an attached 3-liter solvent reservoir at the top and a 4-mm. straight-bore stopcock at the bottom.

To raise the water content, usually 8-9%, of the silicic acid to 13%, spread the portion required in a 0.5 to 1-inch thick layer and distribute the previously determined amount of water evenly throughout the layer with a dropper. Stir thoroughly. Combine all portions and subject to rolling and tumbling in a closed container for 16-24 hours to obtain thorough mixing and equilibrium. Analyze a portion for water content by drying at 110° in an oven for 1 hour and heating at 800° for 1 hour.

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[36] M. A. Phillips and R. D. Hinkel, *J. Agr. Food Chem.* 5, 379-84 (1957).

Stir approximately 30 grams of the prepared silicic acid with 80 ml. of purified cyclohexane to produce a slurry, cover, and let stand for 15 minutes. Place a glass wool plug at the bottom of the 25-mm. section of the column and tamp it into position. Stir the slurry and immediately pour into the column. Wash the beaker with 20 ml. of cyclohexane and add to the column. Connect the column to the source of nitrogen pressure and apply pressure of about 8 pounds per square inch gage to the column. When the solvent level falls to approximately 1 inch above the top of the silicic acid, release the pressure and carefully insert a glass wool plug on top of the silicic acid. Reapply the nitrogen pressure until the solvent level reaches the glass wool plug. Discard all solvent.

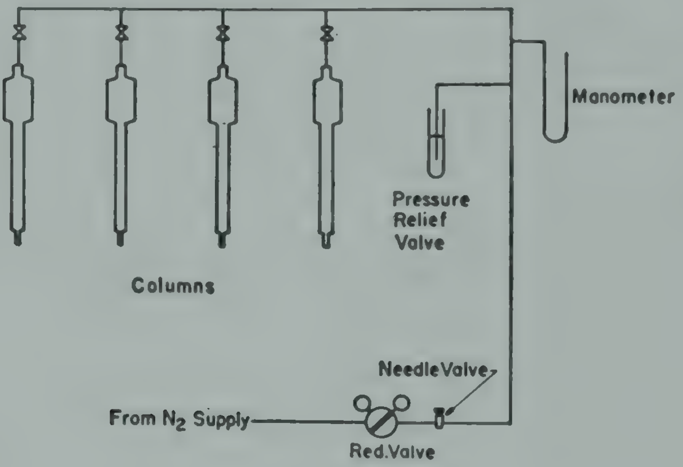

FIG. 5

Diagrammatic sketch of apparatus assembly for 2,6-di-*tert*-butyl-4-methylphenol

Melt a 15-20 gram sample at 100° and dissolve 12.5 grams in purified cyclohexane. Cool to room temperature, dilute to 50 ml. with cyclohexane, and shake well. Release the pressure in the column and introduce 10 ml. of the sample solution close to the top glass-wool plug. Close the column and apply nitrogen pressure. Collect the first filtrate fraction. As soon as the sample solution disappears into the silicic acid, open the column and add 10 ml. of cyclohexane close to the top glass wool plug. When this disappears into the column add another 10-ml. portion. When this disappears into the packing, add enough cyclohexane to fill the reservoir. Collect the filtrate in 50-ml. fractions until at least 6 fractions are obtained. Adjust

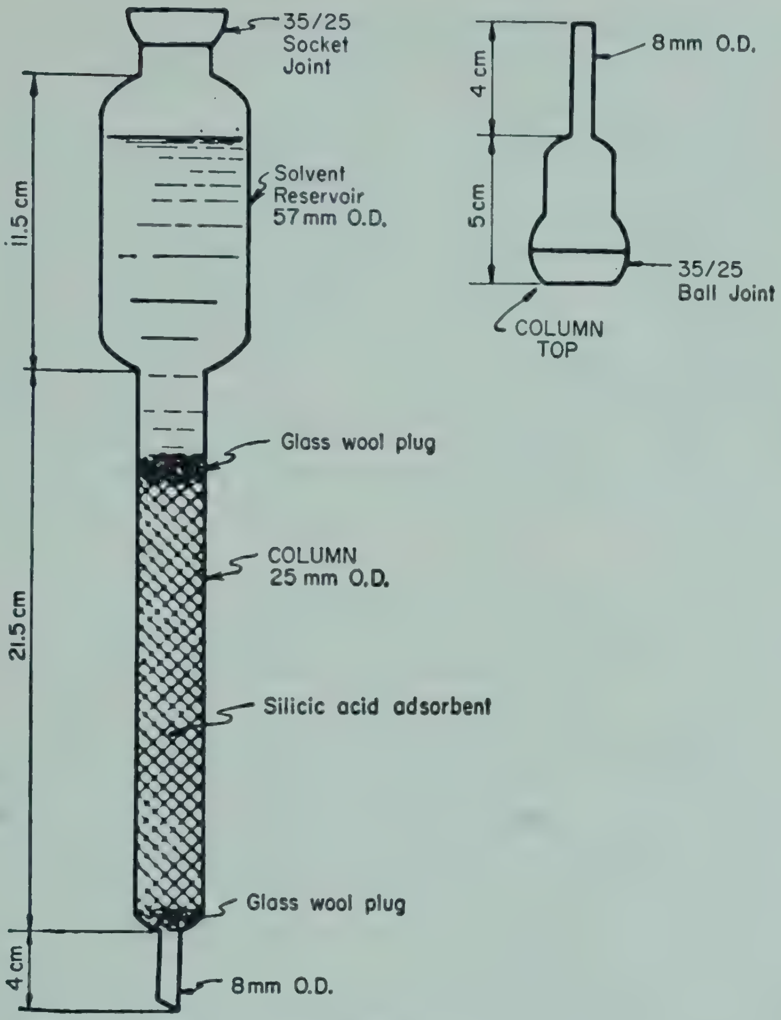
35/25
Socket
Joint
Solvent
Reservoir
57mm O.D.
11.5 cm
Glass wool plug
COLUMN
25 mm O.D.
21.5 cm
Silicic acid adsorbent
Glass wool plug
4 cm
8mm O.D.
8mm O.D.
4 cm
5 cm
35/25
Ball Joint
COLUMN
TOP

FIG. 6

Chromatographic column and top for isolation of 2,6-di-*tert*-butyl-4-methylphenol

the pressure as required to maintain a filtration rate of 2-3 ml. per minute.

Standardize the spectrophotometer over the range of 235-320 mμ with purified cyclohexane. Obtain its absorption curve of each fraction over the range of 235 to 320 mμ. Draw a reference base line on each curve and measure the net absorbance A_N at 284 mμ. Calculate 2,6-di-*tert*-butyl-4-methylphenol as follows:

Grams of 2,6-di-*tert*-butyl-4-methylphenol in a fraction $= G = \frac{A_N}{a'} \times \frac{V_f}{1000}$

where V_f = volume of the filtrate fraction in ml.

a' = absorptivity and A_N is the reading of the fraction.

Vegetable fat shortenings. Increase the adsorbant to 35 grams. There is no interference from as much as 0.08 wt. % of α-tocopherol.

o-DIHYDROXYLBENZENE, CATECHOL, PYROCATECHOL

A photometric method for the determination of catechol (Cf. Vol. III, p. 126) is based on the formation of a yellow coloration with a methanol solution of ethyl amino molybdate, $(C_2H_5NH_3)_2MoO_4$.[37]

The general method for phenols by molybdenum blue (Cf. Vol. III, p. 117) is applicable to catechol and its methyl homologs.[38] The pH must be 2.9-4.5 and the concentration of test substance over 0.005 mg. per ml. The reading is at 435 mμ. Hydroquinone interferes but resorcinol does not. When reacted with titanium sulfate, page 93, the maximum absorption is at 460 mμ. The general class of catecholic phenols is determined by ferrous ion, page 91.

Procedure—*Industrial wastes and phenolic products.* Filter the sample and take an aliquot containing 60-80 mg. of catechol. Dilute with water if necessary, depending on the catechol concentration. Adjust the pH to 6-7 by addition of 1:10 hydrochloric acid or 4% sodium hydroxide solution. Warm to 60° and with stirring add 10% lead acetate solution dropwise so long as more precipitate is formed, plus a few drops in excess. Filter and wash with water. Decompose the precipitate by heating with 1:35 sulfuric acid. Filter, and wash the residual lead sulfate. Dilute the filtrate to a known volume and dilute an aliquot containing 1-4 mg. of catechol to 10 ml. with water.

As reagent neutralize 13.2 grams of ethylamine hydrochloride with a slight excess of 1:1 ammonium hydroxide and add 4.4 grams of molybdenum trioxide. Decant or filter and dilute to 100 ml. Add 5 ml. of a 5% solution of ammonium formate in anhydrous methanol to the sample solution. Add 1 ml. of reagent and read after 3-5 minutes.

p-tert-BUTYL CATECHOL

p-tert-Butyl catechol coupled with diazotized *p*-nitroaniline in basic media gives a red color which is measured spectrophotometrically[39] (Cf. Vol. III, p. 130).

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[37] Jaroslav Janok, *Chem. Listy* 48, 1171-5 (1954).

[38] J. Strachota and Z. Kotášěk, *Ibid.* 52, 1093-8 (1958); *Veda a vyzkum v prumyslu Kozedelnem* 4, 31-48 (1958).

[39] Kalman Marcali, *Anal. Chem.* 27, 1586-94 (1955).

The pH of the solution during extraction and during the coupling reaction must be controlled. If more than 5 ml. of ethanol are added during the procedure, a white precipitate forms and a brownish color develops. More reproducible results are obtained with sodium carbonate than with an alkali metal hydroxide. The absorption maximum is at 466 mμ but a good standard curve for up to 0.002 mg. per ml. is obtained at 525 mμ. A higher sensitivity is obtained at 466 ± 5 mμ. Other aromatic amines and phenols form interfering colors with the reagent.

p-tert-Butyl catechol is an inhibitor in 2-methyl-5-vinylpyridine to prevent polymerization and other reactions during shipment and storage. This decreases in concentration with time. It is determined by air oxidation to the colored quinoid form after extraction from hydrochloric acid solution with diethyl ether and extraction therefrom with sodium hydroxide solution.[40]

In the absence of *o*-aminophenol, another inhibitor sometimes added, *p-tert*-butyl catechol is extracted directly into sodium hydroxide from a carbon tetrachloride solution of the sample. In the first procedure described the *o*-aminophenol is retained in the acid solution of the sample.

Procedure—*By* p-*nitroaniline. Paper treated with elastomers. p-tert-Butyl catechol.* Extract a 900-gram sample with 1800 grams of one of the following solvents chosen to simulate components or conditions of acidity and basicity in food mixtures wrapped in these papers: water; Ringer's solution containing 8.6 grams of sodium chloride, 0.3 gram of potassium chloride, and 0.33 gram of calcium chloride per liter of solution; sodium carbonate solution at pH 10; acetic acid solution at pH 3; 5% lactic acid solution; and 5% sucrose solution. Rotate the paper-solvent mixture in tubes for 7 days at the temperature of the extraction, filter, and determine *p-tert*-butyl catehcol by *p*-nitroaniline.

Other appropriate determinations on this extract are phenothiazine by oxidation with bromine, Resin 731 SA (a disproportionated rosin) by *m*(*p*-dimethylaminophenylazo)benzyltrimethylammonium chloride, and the sodium salt of a condensed mononaphthalenesulfonic acid and the sodium salt of anthraquinonesulfonic acid in acidified ethanol in the ultraviolet.

To prepare the reagent, cool 25 ml. of 0.3% *p*-nitroaniline solution in 1:12 hydrochloric acid in an ice bath for 10-15 minutes. Add 1 ml. of chilled 7.5% sodium nitrite solution, agitate, and store in an ice bath until used.

To a sample containing up to 0.05 mg. of *p-tert*-butyl catechol, add

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[40] K. Nelson and M. D. Grimes, *Ibid.* **30**, 1928-31 (1958).

5 drops of 0.1 *N* acetic acid and gently mix. Extract with two 10-ml. portions of ether. Add 1 ml. of acidified 95% ethanol to the combined extracts to prevent the loss of catechol after the ether is evaporated. Evaporate the ether at 50-55° and add 5 ml. of water to prevent insoluble salt formation after addition of sodium acetate. Add 1 ml. of 31% sodium acetate solution, cool for 10 minutes in an ice bath, and add 1 ml. of reagent. Mix and add 2 ml. of 20% sodium carbonate solution. Agitate for 1 minute in an ice bath, dilute to 25 ml. with water, and read at 525 mμ.

By direct reading. 2-Methyl-5-vinylpyridine. Mix 10 ml. of 1:10 hydrochloric acid, and 5 ml. of diethyl ether with a 1 ml. of sample solution. Shake for 2 minutes to extract the *p-tert*-butyl catechol. Drain the acid phase and re-extract with 5 ml. of diethyl ether for 2 minutes. Shake the combined extracts for 2 minutes with 5 ml. of 1:10 hydrochloric acid. Discard the acid phase. Add 5 ml. of 4% sodium hydroxide solution to the ether and shake for 2 minutes. Dilute the sodium hydroxide phase to 50 ml. with water and read at 485 mμ against water.

m-DIHYDROXYBENZENE, RESORCINOL

The reaction to form a color with iodine which is used for determining either resorcinol or catechol in industrial products (Vol. III, pp. 126-9) is applicable under appropriate conditions to industrial wastes.[41]

The reaction of resorcinol with nitrous acid to form dinitrosoresorcinol is suitable for its estimation (Cf. Vol. III, pp. 127-8).[42] Salicylic acid interferes. Resorcinol is determined by the color reaction with 9-hydroxyxanthene and chloroacetic acid.[43] Resorcinol is also determined by *p*-dimethylaminobenzaldehyde.[44] Reaction with titanium sulfate causes a maximum absorption at 405 mμ, page 93.

Samples—*Phenolic waters.* Neutralize a 25-ml. filtered sample with ammonium sulfate and add 15 ml. of 10% lead acetate solution. Filter off the precipitate and treat the filtrate with 1-2 grams of powdered sodium sulfate. Filter off the lead sulfate and dilute the filtrate to an appropriate volume. Develop by iodine.

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[41] Jaroslav Janak, *Chem. Listy* 48, 1176-80 (1954).

[42] V. Gervay and I. Kelemen, *Acta Pharm. Hung.* 25, 58-67 (1955).

[43] N. P. Yavorskiĭ, *Med. Prom. SSSR* 13, No. 6, 41-3 (1959).

[44] J. Kolšek and M. Perpar, *Z. anal. Chem.* 167, 161-4 (1959).

Phenolic products. Dissolve a 5-10 gram sample in methanol and dilute to 250-500 ml. with water. Filter a 100-150-ml. aliquot and dilute a 25-ml. aliquot to 250-500 ml. Develop a 2-5-ml. aliquot by iodine.

Ointments. Reflux a 50-100-mg. sample with 10 ml. of 95% ethanol for 5 minutes. Cool and decant through a filter. Repeat the extraction by similar refluxing with 5 ml. and 5 ml. of 95% ethanol decanting through the same filter. Wash the filter with 95% ethanol and dilute the filtrate to 25 ml. with ethanol. Develop with 9-hydroxyxanthene and chloroacetic acid.

Procedure—*By iodine.* Mix a portion of sample solution containing 0.05-0.2 mg. of resorcinol with 2.5 ml. of 0.2% catechol solution. Dilute to 10 ml. and add 10 ml. of acetate buffer, prepared by adding 30 ml. of acetic acid to 800 ml. of water, and neutralizing with 75 ml. of 25% sodium hydroxide solution. Add 15 ml. of 1.3% iodine solution. After 1 minute, destroy the excess iodine with 15 ml. of 2.5% sodium thiosulfate solution and dilute to 50 ml. with acetone. Read at 520 mμ.

By nitrous acid. Dilute an aqueous sample containing 1-3 mg. of resorcinol to 15 ml. Add 4 ml. of 3% acetic acid and 3 ml. of 0.5% sodium nitrite solution. Dilute to 25 ml. with water and maintain at 20-25° for 1.5 hours. The solution becomes blue with dinitrosoresorcinol monohydrate. Read at 450 mμ.

By dimethylaminobenzaldehyde. Resorcinol or phloroglucinol. To 2 ml. of sample solution containing up to 0.1 mg. of either, add 2 ml. of a reagent containing 0.5 g. of *p*-dimethylaminobenzaldehyde in 17 ml. of 1:2 sulfuric acid. After 30 minutes read at 518 mμ for resorcinol. Read after 30 minutes at 550 mμ for phloroglucinol.

By 9-hydroxyxanthene and chloroacetic acid. As reagent dissolve 25 grams of chloroacetic acid in the minimum volume of 95% ethanol at 30°. Add 0.2-0.3 gram of activated carbon. After 20-30 minutes filter and make to 50 ml. with 95% ethanol. Use the day prepared.

To 5 ml. of sample in ethanol add 9 ml. of fresh 5% solution of 9-hydroxyxanthene and 10 ml. of the chloroacetic acid reagent. Dilute to 25 ml. with ethanol. Reflux for 20 minutes and cool. Read within 35 minutes at 590 mμ.

5-METHYL-2-ISOPROPYL-1-PHENOL, THYMOL

When thymol is treated with dimethylaminobenzaldehyde in sulfuric acid, a yellow color develops.[45] The sulfuric acid as a solvent for the reagent is the factor producing reproducible, stable results. The uniformity of shaking or time interval does not affect results. Linalool, α-pinene, *p*-pinene, *p*-cymene, geraniol, and borneol, normally present in thyme oil, interfere only if present in unusually high concentrations. Beer's law is followed.

When thymol is reacted with titanium sulfate the maximum absorption is at 438 mμ, page 93. The maximum fluorescence at pH 7 is at 300 mμ.[46]

Procedure—Dilute a 100-mg. sample of thyme oil, approximately 3-4 drops, to 50 ml. with chloroform. Dilute a 1-ml. aliquot to 25 ml. with chloroform. To an aliquot of this chloroform solution containing 0.0088-0.044 mg. of thymol, add 5 ml. of 0.125% *p*-dimethylaminobenzaldehyde solution in concentrated sulfuric acid. Stopper and shake briskly for 10 seconds. Read at 400-465 mμ. Correct for a reagent blank containing chloroform in place of the sample.

THYMOL AND CARVACROL

Diazotized sulfanilic acid develops a color with the phenolic group of thymol (Vol. III, p. 122) and carvacrol.[47]

Procedure—*Thyme.* Steam-distil the sample from an acid solution and collect in 20% sodium hydroxide solution. Dilute so that the final solution contains 2% of sodium hydroxide and 0.02-0.04 mg. of thymol and carvacrol per ml. Add 3 ml. of sample solution to a mixture containing 4 ml. of 0.5% sulfanilic acid solution, 1 ml. of 1:49 sulfuric acid, and 2 ml. of 0.5% sodium nitrite solution. Read after 1 minute at 470 mμ.

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[45] L. Fibranz, M. I. Blake, and C. E. Miller, *J. Am. Pharm. Assoc.* **47**, 133-5 (1958).

[46] S. Udenfriend, D. E. Duggan, B. M. Vasta, and B. B. Brodie, *J. Pharmacol. Exptl. Therap.* **120**, 26-32 (1957).

[47] U. Bogs and J. Meinhard, *Arch. Pharm.* **288**, 134-9 (1955).

l-α-3,4-Dihydroxy-α (methylaminomethyl)benzyl alcohol
Epinephrine, Adrenaline

Oxidation of adrenaline with various reagents (Vol. III, pp. 130-6) including potassium ferricyanide,[48] potassium permanganate,[49] iodine,[50] mercuric nitrate,[51] and dipotassium mercuric tetrathiocyanate,[52] iodate,[53] persulfate,[54] or manganese dioxide[55] followed by the measuring of the fluorescence of the resulting adrenochrome remains the principal method of determination.

Ferricyanide and zinc sulfate oxidize adrenaline in 3 minutes under the conditions described in the procedure at pH 3.5 and noradrenaline is oxidized less than 5% at pH 3.5. An acetate buffer is used. Large amounts of ascorbic acid cause quenching. Compounds which possess a hydroxyl group at the β-carbon and hydrogen at the α-carbon in the side chain cause fluorescence. Thus, isopropylnoradrenaline gives strong fluorescence. In oxidation with permanganate it is possiple to distinguish adrenaline from noradrenaline by adding 20% sodium hydroxide solution to the oxidation products. This causes the noradrenaline to develop later with less intensity than adrenaline.

Adrenaline and noradrenaline are determined by permanganate oxidation. At pH 3.6 adrenaline is completely oxidized in 2 minutes while less than 10% of noradrenaline is oxidized during the same time. Both compounds are oxidized completely within 3 minutes at pH 5-6.[56]

Noradrenachrome and adrenachrome are differentially transformed [57] for reading fluorescently. Thus after treatment in an alkaline medium, on addition of acid adrenochrome is oxidized to adrenolutine almost instantly but noradrenochrome relative slowly to noradrenolutine. The lutines are not fluorescent.

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[48] E. Popper, V. Spahl, and N. Ariton, *Acad. rep. populare Române, Filiala Cluj, Studii cercetări stiint.* **3**, 36-41 (1952); U. S. von Euler and I. Floding, *Acta Physiol. Scand.* **33**, Suppl. 118, 45-56 (1955); *Scand. J. Clin. and Lab. Invest.* **8**, 288-95 (1956); W. J. Griffiths and S. Collinson, *J. Clin. Pathol.* **10**, 120-5 (1957).

[49] T. Suzuki and T. Ozaki, *Tohoku J. Exptl. Med.* **54**, 332 (1951); A. Pekkarinen and M. E. Pitkanen, *Scand. J. Clin. and Lab. Invest.* **7**, 1-7 (1955).

[50] A. Correia Alves, *Anais fac. farm. Porto* **12**, 41-50, 63-78 (1952); E. Egger and F. Lorenz, *Z. physiol. Chem.* **311**, 96-100 (1958).

[51] J. van Espen, *J. pharm. Belg.* **14**, 56-66 (1959).

[52] E. Salgo, J. Kis, and J. Morvay, *Z. anal. Chem.* **149**, 355-8 (1956).

[53] F. G. Stock and L. W. Hinson, *J. Pharm. and Pharmacol.* **7**, 512-21 (1955).

[54] A. Pekkarinen and M. E. Pitkanen, *Scand. J. Clin. and Lab. Invest.* **7**, 1-7 (1955).

[55] F. Bischoff and A. Torres, *J. Appl. Physiol.* **14**, 237-40 (1959).

[56] T. Suzuki and T. Ozaki, *Tohoku J. Exptl. Med.* **54**, 332 (1951).

[57] S. Roston and S. Blumberg, *U. S. Army Med. Research Lab., Rept.* **No. 295**, 12 pp. (1957); Sidney Roston, *Anal. Chem.* **30**, 1363-6 (1958).

The position of the visible absorption peak when adrenalin reacts with iodine depends upon time. In 20 minutes, at pH 4.8, the maximum is brought from 492 to 520-2 mμ and at pH 7.2 it is brought from 492 to 532-4 mμ within 1 hour. At pH 4.8, 30% ethanol solution enhances the peak and delays its shift while at pH 7.2 it only enhances the peak. The first peak obtained belongs to adrenochrome and the latter to 2-iodo-3-hydroxy-1-methyl-2,3-dihydroindole-5,6-quinone.[58] The oxidation products also have peaks at 347 mμ for adrenaline and at 350 mμ for noradrenaline.[59] Separation by paper chromatography before oxidation is necessary.

Preoxidation of adrenaline as well as noradrenaline by the catalytic action of tyrosinase increases the effectiveness of the fluorescence method.[60] Between pH 1.88 and 6.70, adrenaline exhibits a large absorption maximum at 279.5 mμ and a small one at 221 mμ. The extinction coefficient at 279.5 mμ depends on pH, except at pH 4.7-5.7.[61]

One determination of adrenaline and noradrenaline is based on the fluorimetric reading of the product obtained by the condensation of these compounds with ethylene diamine.[62] Ammonium molybdate is a catalyst for the condensation.[63] A related reaction is condensation with 2,3-diaminonaphthalene.[64] Since the fluorescence emission of the adrenaline-ethylenediamine compound on excitation with light of 436 mμ is almost twice that obtained with light of 365 mμ, and the fluorescence of the noradrenaline-ethylenediamine compound shows no increase with these changes, adrenaline and noradrenaline can be quantitatively differentiated. A system of primary and secondary filters is therefore required to determine 0.00005 mg. of adrenaline and 0.0002 mg. of noradrenaline. Adrenaline can be distinguished from riboflavin which gives a similar reaction by the ease with which adrenaline is destroyed by potassium permanganate.

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[58] A. Correia Alves, *Anais fac. farm. Porto* 12, 63-78 (1952).

[59] R. L. Tse and M. J. Oesterling, *Clin. Chim. Acta* 4, 307-9 (1959).

[60] E. Mylon and S. Roston, *Am. J. Physiol.* 172, 612-6 (1953).

[61] A. Correia Alves, *Anais fac. farm. Porto* 12, 41-50 (1952).

[62] S. Natelson, J. K. Lugovory, and J. B. Pincus, *Arch. Biochem.* 23, 157-8 (1949); H. Weil-Malherbe and A. D. Bone, *Biochem. J.* 51, 311-8 (1952); H. Persky and S. Roston, *Science* 118, 381-2 (1953); J. A. Richardson, A. K. Richardson, and O. J. Brodie, *J. Lab. Clin. Med.* 47, 832-4 (1956); W. J. Griffiths and S. Collinson, *J. Clin. Pathol.* 10, 120-5 (1957).

[63] K. Erne and T. Canbäck, *J. Pharm. and Pharmacol.* 7, 248-54 (1955).

[64] Hirsh Sulkowich, *Entocrinology* 59, 260-2 (1956).

In the determination of adrenaline by phospho-18-tungstic acid (Vol. II, p. 137), uric acid is used as a reference standard. The colorimetric atio of uric acid to adrenaline is 1:2.97 to 1:3.03. If the adrenaline conains 10, 20, or 30% of noradrenaline, the ratio is 1:2.85, 1:2.67, and :2.39 respectively.[65]

Up to 0.0005 mg. per ml. of adrenaline or noradrenaline can be etermined by the stable deep-red color formed by treating with sodium itrite in acid solution followed by making the solution basic. Adrenalone ives the same reaction but ephedrine, sympatol, and similar compounds lo not react.[66]

Colored compounds are formed by the reaction of adrenaline with odium nitroprusside-formaldehyde and secondary amines in alkaline olution.[67] Interference by ephedrine and procaine is avoidable.

Adrenaline, as well as corbasil, noradrenaline, and novodrin, can be letermined in the presence of procaine, Pantocaine, and falicaine by he violet color formed by adrenaline and ferrous ion is buffered hydrochloric acid solutions.[68]

Ferric ammonium oxalate gives a bluish-violet with adrenaline or adrenal gland extract which is stable for 24 hours.[69] While the reading at 550 mμ is quantitative, and uric acid, ascorbic acid, etc. do not interfere, it is not applicable to blood or drug preparations.

To determine adrenaline in the presence of sodium bisulfite, sodium metabisulfite, or other reducing agents which serve as stabilizers for injectable solutions of adrenaline, treat the solution with ammonium hydroxide, precipitate the sulfite with hot barium acetate solution, and determine with potassium ferricyanide.[70]

By addition of sodium metabisulfite and sodium fluoride the content of adrenaline and noradrenaline can be preserved in urine for 7 days at room temperature.[71] Adrenaline and noradrenaline are separable chromatographically.[72] They are also sorbed by appropriate ion-exchange resins after which they can be eluted and determined.[73] Commercial aluminum oxides[74] used as sorbants often contain fluorescent contaminants which must be removed.[75] For sorption on aluminum oxide a pH of 8.5 is desirable followed by elution with 1:140 sulfuric acid.[76]

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[65] Toshiyuki Ozaki, *Tohoku J. Exptl. Med.* **61,** 322 (1955).

[66] R. Reiss, *Z. anal. Chem.* **145,** 265-8 (1955).

[67] B. Majumdar and S. B. Sengupta, *Indian J. Pharm.* **15,** 260-2 (1953).

[68] J. Richter, *Z. anal. Chem.* **145,** 334-6 (1955).

[69] R. G. Roberts and D. M. Hilker, *Proc. Soc. Exptl. Biol. Med.* **82,** 556-8 (1953).

[70] E. Popper, V. Spahl, and N. Ariton, *Acad. rep. populare Române, Filiala Cluj, Studii cercetări științ.* **3,** 36-41 (1952).

[71] Charles Sobel and Richard J. Henry, *Tech. Bull. Registry Med. Technologists* **27,** 18-23 (1957); *Am. J. Clin. Pathol.* **27,** 240-5 (1957).

[72] M. S. Filbert and M. Cornblath, *J. Appl. Physiol.* **8,** 59-66 (1955).

[73] T. B. B. Crawford and W. Low, *J. Pharm. and Pharmacol.* **10,** 179-88 (1958).

The catecholamines are adrenaline, noradrenaline, and 3-hydroxytyramine. All three are developed by condensation with ethylenediamine.[77] The adrenaline and noradrenaline are separately determined [78] and subtracted. Likewise 3-hydroxytyramine is separately determined and subtracted.

When the catechol amines are extracted with 4% perchloric acid solution and the extracts passed through a column of Dowex 50, this separates the amines which are eluted with hydrochloric acid.[79] After oxidation the activation peaks and fluorescence peaks are respectively adrenolutine 425 mμ and 345 mμ, noradrenolutine 410 mμ and 535 mμ. At pH 1, the maximum fluorescence of adrenaline or noradrenaline is emitted at 335 mμ.[80]

Samples—*Urine*.[81] To prepare alumina for sorption boil 200 grams of aluminum oxide in 1 liter of 1:5 hydrochloric acid. Filter with suction, wash with water, and dry.

Adjust a 0.1-ml. portion of a 24-hour sample to pH 3, heat to boiling, and filter. To the filtrate add 2.5 grams of the acid-washed alumina and 1 gram of ethylenediaminetetraacetic acid. With vigorous stirring add 2% sodium hydroxide solution to raise the pH to 8.5. Filter rapidly and wash twice with 10-ml. portions of water. Elute the adrenaline from the alumina with two 4.5-ml. portions of 1:143 sulfuric acid and collect the luates in a flask containing 0.05 ml. of 0.04% bromophenol blue solution. eutralize with 10% sodium bicarbonate solution to pH 4 and dilute o 10 ml. This solution can be stored in a refrigerator for 1 week or rozen for months. Determine adrenaline and noradrenaline with potasium ferricyanide.

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[74] R. Robinson and F. D. Scott, *Biochem. J.* 68, 28P (1958).

[75] R. T. Jones and W. D. Blake, *J. Appl. Physiol.* 12, 448-52 (1958); Cf. C. H. Brilmayer, H. H. Edck, and M. Littig, *Med. Klin. (Munich)* 52, 1184-7 (1957); Cf. N. A. Smazhnova, *Sbornik Nauch. Rabot Sotrudnikov Tsentral Nauch.-Issledovatel. Lab., 2-oĭ Moskov. Med. Inst.* 1958, No. 1, 66-73.

[76] P. Gonnard and A. Pelon, *Ann. biol. clin. (Paris)* 16, 579-85 (1958).

[77] H. Weil-Malherbe and A. D. Bone, *Biochem. J.* 51, 311-18 (1952); *J. Clin. Pathol.* 10, 138-47 (1957); Cf. Arthur de T. Valk, Jr., and Henry L. Price, *J. Clin. Invest.* 35, 837-41 (1956).

[78] U. S. von Euler and I. Orwén, *Acta Physiol. Scand.* 33, Suppl. 118, 1-10 (1955); U. S. von Euler and I. Floding, *Ibid.* 33, Suppl. 118, 57-62 (1955).

[79] Å. Bertler, A. Carlsson, and E. Rosengren, *Acta Physiol. Scand.* 44, 273-92 (1958); Å Bertler, A. Carlsson, E. Rosengren, and B. Waldeck, *Kgl. Fysiograf. Sällskap. Lund, Förh.* 28, 121-3 (1958); Cf. Å. Bertler, A. Carlsson, and E. Rosengren, *Clin. Chim. Acta* 4, 456-7 (1959).

[80] S. Udenfriend, D. E. Duggan, B. M. Vasta, and B. B. Brodie, *J. Pharmacol. Exptl. Therap.* 120, 26-32 (1957).

[81] U. S. von Euler and I. Floding, *Scand. J. Clin. and Lab. Invest.* 8, 288-95 (1956).

Alternatively,[82] adjust the pH of an acidified 24-hour urine sample to H 8.5, filter the precipitated phosphates, and add 8 grams of chromotoraphic-grade alumina. Mix in a blender and centrifuge. Discard the upernatant liquid and wash the precipitate 5 times with 100-ml. portions f water. Elute the adrenaline and noradrenaline with 20 ml. of 4.5% xalic acid. After several hours, remove the precipitated calcium oxalate nd adjust the pH to 6.3 with 8% sodium hydroxide solution. Stabilize y adding 10 ml. of 16% sodium acetate solution. Dilute to 200 ml. and etermine adrenaline and noradrenaline by oxidation with permanganate.

Blood.[83] To prepare the reagent, dissolve 2 grams of sodium fluoride and 3 grams of sodium sulfite in 100 ml. of water. Autoclave and put up in 6-ml. ampules. Draw the blood sample with a syringe containing 5 ml. of the reagent. Separate the plasma by centrifuging. Add an equal volume of sodium acetate buffer for pH 8.4. Filter through a column of acid-washed alumina and wash with water. Elute the adsorbed adrenaline with dilute acetic acid. Determine with ethylenediamine.

Procedure—*Adrenaline and noradrenaline by manganese dioxide.*[84] To 6 ml. of sample solution containing 0.01-0.02 mg. of adrenaline or noradrenaline, add 10 mg. of manganese dioxide and mix for 30 seconds. Centrifuge and filter. Treat 2 ml. of the filtrate with 0.4 ml. of 20% sodium hydroxide solution containing 200 mg. of ascorbic acid per 100 ml. Measure the fluorescence using No. 5860 and 3389 filters against a blank treated with sodium hydroxide without ascorbic acid. The result is adrenaline and noradrenaline. Repeat the determination without ascorbic acid in the 20% sodium hydroxide solution. The result is adrenaline. Noradrenaline only develops later.

Adrenaline and noradrenaline by permanganate.[85] By application of a factor for the differential rate of oxidation as affected by pH, each is calculated. Adjust the pH of a 1-ml. sample to 3.6 and of another 1-ml. sample to 5.6 with sodium acetate-acetic acid buffer. To each add 0.1 ml. of potassium permanganate solution prepared from 3 grams of potassium permanganate, 24 ml. of water, and 8 ml. of lactic acid. After 2 minutes add 0.1 ml. of 3% hydrogen peroxide solution to the solution at pH 3.6 to stop the reaction. After 3 minutes add 0.1 ml. of 3% hydrogen peroxide to the solution at pH 5.6. Dilute to known volumes and read the color. Calculate arterenol and adrenaline as follows:

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[82] A. Pekkarinen and M. E. Pitkanen, *Ibid.* **7,** 1-7 (1955).

[83] H. Weil-Malherbe and A. D. Bone, *Biochem. J.* **51,** 311-8 (1952); Cf. A. F. de Schaepdryver, *Arch. intern. pharmacodynamie* **117,** 475-84 (1958).

[84] A. Pekkarinen and M. E. Pitkanen, *Scand. J. Clin. and Lab. Invest.* **7,** 1-7 (1955).

[85] T. Suzuki and T. Ozaki, *Tohoku J. Exptl. Med.* **54,** 332 (1951); Toshiyuko Ozaki, *Ibid.* **61,** 83-92 (1954).

$$A = x + n_{3.6}y$$

$$B = x + n_{5.6}y$$

where

x = the amount of adrenaline

y = the amount of noradrenaline

A = adrenaline equivalent estimated at pH 3.6

B = adrenaline equivalent estimated at pH 5.6

$n_{3.6}$ = the ratio of adrenaline:noradrenaline necessary to form the same color density after oxidation at pH 3.6 in 2 minutes

$n_{5.6}$ = the ratio of adrenaline:noradrenaline necessary to form the same color density after oxidation at pH 5.6 in 3 minutes.

Adrenaline and noradrenaline by ferricyanide.[86] *Adrenaline alone.* To a solution containing 0.00002-0.0005 mg. of adrenaline, add 1 ml. of *M* acetate buffer for pH 3.5. Follow by 0.1 ml. of 0.25% potassium ferricyanide solution and 0.1 ml. of 0.5% zinc sulfate solution. Allow 3 minutes to oxidize the adrenaline and add 1 ml. of a 9:1 mixture of 20% sodium hydroxide solution and 2% ascorbic acid solution. Read against a blank.

As the blank treat a duplicate sample with 0.1 ml. of 0.25% potassium ferricyanide solution and 0.9 ml. of 20% sodium hydroxide solution and allowing 2 minutes for fluorescence to fade. This blank is stable for at least an hour.

Adrenaline and noradrenaline. To a solution containing 0.00002-0.0005 mg. of total adrenaline and noradrenaline, add 1 ml. of *M* acetic acid-sodium acetate buffer for pH 6.0 and 0.1 ml. of 0.25% potassium ferricyanide solution. After 2 minutes add 1 ml. of a 9:1 mixture of 20% sodium hydroxide solution and 2% ascorbic acid solution. Dilute to 10 ml., mix and read the fluorescence 5-60 minutes later against a blank prepared as for adrenaline alone.

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[86] U. S. von Euler and I. Floding, *Acta. Physiol. Scand.* 33, Suppl. 118, 45-56 (1955).

As an alternative for adrenaline and noradrenaline[87] take two 2-ml. portions of sample. To each add 1 ml. of *M* acetate buffer for pH 5.5 and 1 ml. of 0.25% potassium ferricyanide solution. These are 1 and 2. While this proceeds for 5 minutes at 25° prepare 3 tubes. Tube 3 contains 0.75 ml. of 20% sodium hydroxide solution and 5.9 ml. of water. Tube 4 contains 0.25 ml. of 0.08% ascorbic acid solution. Tube 5 contains 4 ml. of 0.08% ascorbic acid solution, 0.75 ml. of 20% sodium hydroxide solution, and 2.15 ml. of water.

To determine noradrenaline, mix Tube 1 with Tube 3. After 30 seconds add the contents of Tube 4. Read the fluorescence after 5 minutes.

To determine adrenaline, mix Tubes 2 and 5 and read the fluorescence after 5 minutes.

Adrenaline by dipotassium mercuric tetrathiocyanate.[88] Dissolve a 2-3 mg. sample in 50 ml. of water at 40°-45° and dilute to 100 ml. with water at 20°. Treat an aliquot containing 0.1-0.15 mg. of adrenaline with 2 ml. of 2.5% dipotassium mercuric tetrathiocyanate solution. Heat at 100° for 25 minutes and cool. Dilute to 10 ml. and read at 500 mμ against a reagent blank.

Adrenaline by sodium nitroprusside.[89] Treat the sample solution with 0.2 ml. of 1% sodium nitroprusside solution containing 4% of formaldehyde. Dilute to 10 ml. with water. If ephedrine hydrochloride is present, add 5 ml. of 1% sodium carbonate solution. If procaine hydrochloride is present, add 2 ml. of 1% sodium bicarbonate solution. If neither is present add 1 ml. of 1% sodium carbonate solution. Read after 30 minutes with a blue filter.

Adrenaline, noradrenaline, and 3-hydroxytyramine by ethylenediamine.[90] *Blood.* As reagent prepare 2% sodium fluoride and 3% sodium thiosulfate solution and autoclave. Withdraw 15 ml. of blood into a syringe containing 5 ml. of reagent. Centrifuge and add an equal volume of sodium acetate-acetic acid buffer for pH 8.4. Filter through a column of acid-washed alumina and wash. Elute the amines with dilute acetic acid. Add 0.5 ml. of 27% ethylenediamine dihydrochloride solution and 0.7 ml. of ethylenediamine. Heat at 50° for 20 minutes. Cool and saturate with sodium chloride. Extract with 6 ml. of isobutanol and measure the fluorescence. Compare with a standard of 0.0002 mg. of adrenaline and a reagent blank.

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[87] Sidney Roston, *Anal. Chem.* **30,** 1363-6 (1958).

[88] E. Salgo, J. Kis, and J. Morvay, *Z. anal. Chem.* **149,** 355-8 (1956).

[89] B. Majumdar and S. B. Sengupta, *Indian J. Pharm.* **15,** 260-2 (1953).

[90] H. Weil-Malherbe and A. D. Bone, *Biochem. J.* **51,** 311-8 (1952); **67,** 65-72 (1957).

α-NAPHTHOL

A mixture of two organic compounds containing the same functional group can be analyzed by their differences in rate of reaction with a third substance.[91] So the faster rate of reaction of α-naphthol than β-naphthol with diazotized 2-naphthylamine-5, 7-disulfonic acid permits the determination of up to 0.5% of α-naphthol in the presence of β-naphthol (Cf. Vol. III, pp. 144-5).[92] The β-naphthol content of the sample is reduced prior to colorimetric development by precipitation wth acid, and an excess of reagent is required to prevent the interference of the remaining β-naphthol.

A 0.5N hydrochloric acid concentration is chosen since the effect of small changes in acidity on the absorbancy is less critical. It is easy to adjust the solution to this hydrochloric acid concentration, and the interferences of β-naphthol and the effect of time on the reaction are decreased. Prolonged heating at the 70°-80° temperature required in the procedure should be avoided. In order for the temperature error to be less than 0.1 mg. of α-naphthol, the temperature must be controlled at ±0.5°.

Sunlight and other variations above normal laboratory lighting may give low results, although not significantly large. It is possible that the light affects the inner conversion of the *cis-trans* isomerism of the azo compound. β-Thionaphthol and β,β′-dinaphthyl disulfide do not interfere. Since the color formation depends upon reaction rate, catalysts interfere. Beer's law is followed for 1-4 mg. of α-naphthol at 485 mμ.

When napalm, a mixture of soaps of aluminum made from oleic acids, naphthenic acids, and other acids, is to be analyzed, the diazotization and coupling method to determine α-naphthol is ineffective because of interferences from naphthenic acids and phenolics present. Sodium cupribromide, which forms a blue or violet color with α-naphthol, is then a suitable reagent.[93]

Catechol gives a brown color with the reagent which can be differentiated spectrophotometrically from the blue of the α-naphthol complex. Phenol, 1,8-dihydroxynaphthalene-3,6-disulfonic acid, β-naphthol, 1-naphthol-2-sulfonic acid, 2,7-dihydroxynaphthalene, 1,5-dihydroxynaphthalene, and 1,1′-bi-2-naphthol show either little color or extremely poor sensitivity. The method is suitable for determining 0.001 mg. of α-naphthol per ml. of solution in the presence of similar compounds.

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[91] T. S. Lee and I. M. Kolthoff, *Ann. N. Y. Acad. Sci.* **53**, 1093-1107 (1951).

[92] J. S. Parsons, W. Seaman, and J. T. Woods, *Anal. Chem.* **27**, 21-24 (1955).

[93] A. Sa and A. D. Marsico, *Anales asoc. quím. argentina* **31**, 202-12 (1943); S. Sass, J. J. Kaufman, and J. Kiernan, *Anal. Chem.* **29**, 143-4 (1957).

A fluorometric method for the determination of either α-naphthol or β-naphthol in the range of 0.15-18 ppm. in alkaline solution is possible without prior separation of the naphthols.[94] Although ultraviolet fluorescence is observed for β-naphthol in acidic aqueous solutions and both compounds show ultraviolet fluorescence in methanol, visible fluorescence is used because of a smaller degree of overlap of the spectra. A calibration curve is necessary regardless of the wave length employed since there is a mutual interference between the two naphthols, which results in some degree of quenching. At a pH above 10 the fluorescence is independent of pH and therefore 13 is chosen. Little loss of fluorescence is seen after 30 minutes, but a decrease of 10% is observed after 8 hours. Both photooxidation and photodegradation occur, but the presence of oxygen, small temperature changes, and moderate exposure to ultraviolet radiation does not cause significant changes.

Because of the variations of source intensity with time, standardization of the spectrophotometer with a quinine solution is necessary before each series of measurements. Naphtholcarboxylic acids, naphtholsulfonic acids, and many naphthalenediols which fluoresce in alkaline solution interfere. A 30-fold excess of naphthalene, 2-chloronaphthalene, and naphthalene 2-sulfonic acid do not interfere. When reacted with titanium sulfate, the absorption maxima for α-naphthol are at 440 and 665 mμ, page 93.

Samples—*β-Naphthol.* Dissolve a 1-gram sample in 10 ml. of 4% sodium hydroxide solution, warming if needed. Add 50 ml. of water and heat to 70°-80°. Slowly add 9 ml. of 1:1 hydrochloric acid with constant swirling to precipitate most of the β-naphthol. Cool in an ice bath at 2°. Allow to stand for 10 minutes and filter with suction. Wash out the reaction flask with 5 ml. of ice water and cool the washings in the ice bath. Add to the precipitate when it is practically free from liquid and suck through. Dilute the filtrate to 100 ml. and develop with diazotized 2-naphthyl-amine-5,7-disulfonic acid.

Napalm. Disperse by shaking a 0.5-gram sample containing 0.01-0.05 mg. of α-naphtol in 25 ml. of a mixed solvent containing 5 parts of isooctane, 5 parts of toluene, and 3 parts of absolute ethanol. Develop with sodium cupribromide.

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[94] D. M. Hercules and L. B. Rogers, *Anal. Chem.* 30, 96-9 (1958).

Procedure—*By diazotized 2-naphthylamine-5,7-disulfonic acid.* To prepare the reagent, recrystallize the monosodium salt of 2-naphthylamine-5,7-disulfonic acid from water several times with the addition of decolorizing carbon until the product is no more than faintly yellow. Dry in a steam oven and titrate with 0.1 *N* sodium nitrite to an external starch-iodide and point to establish the amount of nitrite necessary to prepare the reagent solution. Excess nitrite is undesirable.

Dissolve an amount of the recrystallized salt equivalent to 0.325 gram of the monosodium salt of 2-naphthylamine-5,7-disulfonic acid in 25 ml. of 1:1 hydrochloric acid. Cool to 10° and place in an ice bath. Add 10 ml. of 0.1 *N* sodium nitrite solution slowly with stirring and dilute to 50 ml. with ice water. Keep in an ice bath and prepare fresh daily. Place the 100-ml. sample containing 1-4 mg. of α-naphthol in a bath at 23°-24°. When the aliquot reaches the temperature of the bath, add 4 ml. of reagent, starting a stop watch as soon as the flow of reagent begins. Shake the solution occasionally, but do not expose to sunlight or other strong light. Remove after 13 minutes and read at 485 mμ against water exactly 15 ± 0.1 minutes from the beginning of the timing period. For the calibration curve an amount of β-naphthol equivalent to that in the prepared sample must be present with variable amounts of α-naphthol.

By sodium cupribromide.[95] To a dissolved sample containing 0.01-0.05 mg. of α-naphthol, add 1 ml. of sodium cupribromide reagent containing 0.4 gram of sodium bromide and 0.01 gram of cupric chloride in water. Shake and filter. Dilute to 10 ml. and read within 5 minutes at 510 mμ. As a reagent blank treat a 0.5-gram α-naphthol-free sample similarly.

By fluorescence.[96] Dissolve a solid sample to give a final concentration of 0.015-0.0015 mg. per ml. in a mixture containing 20 parts of 95% ethanol, 20 parts of 4% sodium hydroxide solution, and 60 parts of water. If the sample is liquid, dilute so that the final 95% ethanol volume is 2 ml. Add 2 ml. of 4% sodium hydroxide solution and dilute to 10 ml. with water.

In either case let stand to reach room temperature. Prepare three silica cells, two containing 3 ml. of the sample and one containing 3 ml. of a quinine reference solution. Adjust the sensitivity of the spectrophotometer with a 3-ppm. quinine reference solution so that the transmittance dial reads 30.0% *T*, read directly in quinine equivalents times 10. Excite fluorescence by 365 mμ radiation and read at 480 mμ for α-naphthol and 426 mμ for β-naphthol.

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[95] S. Sass, J. J. Kaufman, and J. Kiernan, *Ibid.* 29, 143-4 (1957).

[96] D. M. Hercules and L. B. Rogers, *Ibid.* 30, 96-9 (1958).

COUMARIN

p-Nitroaniline (Vol. III, p. 150) is a reagent for determination of coumarin. It may also be determined by direct reading at 270 mμ after separation from vanillin and ethyl vanillin by chromatography on silicic acid.[97] As given, the method includes separation and reading of vanillin and ethylvanillin at 270 mμ. Coumarin is also read directly at 335 mμ.[98]

Procedure—*Vanilla extract. Direct reading.* To a 25-ml. sample, add 75 ml. of water, 20 ml. of 1:4 sulfuric acid, and 50 ml. of chloroform. Stopper and shake for 3 minutes. Centrifuge for 5 minutes. If emulsion persists, break with a glass rod and recentrifuge. Pour slowly through a large-bore short-stem funnel into a separatory funnel. Break any emulsion with a glass rod and remove the chloroform layer. Re-extract the aqueous phase with three 15-ml. portions of chloroform and recentrifuge. Dilute the combined chloroform layers with isooctane to 100 ml. Dilute a 2-ml. aliquot to 25 ml. with isooctane.

To prepare the isooctane-chloroform solvent mixture for chromatography, add 40 ml. of chloroform to 1 liter of isooctane and mix. Wash with 500 ml. of water and discard the aqueous phase. Filter through cotton.

To prepare the sorbant, mix 4 grams of 100-mesh silicic acid with 2.2 ml. of water to a uniform powdery consistency and immediately add 25 ml. of solvent. Mix and pour rapidly into a 18-inch chromatographic tube with a 12.5 mm. inside diameter, constricted at one end. The base contains glass beads and a small wad of cotton. Remove any air bubbles with a glass rod. Pack the column with 2 lb. per square inch pressure until the top surface is just firm and immediately release the pressure. If the column channels or cracks, discard. Add 15 ml. of solvent without disturbing the column and force through the column. It is now ready for calibration.

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[97] J. G. Kirchner, J. M. Miller, and G. J. Keller, *Anal. Chem.* 23, 420-5 (1951); J. M. Miller and J. G. Kirchner, *Ibid.* 26, 2002 (1954); Luther G. Ensminger, *J. Assoc. Offic. Agr. Chemists* 36, 679-97 (1953); *Ibid.* 38, 730-8 (1955); Official Methods of the Association of Official Agricultural Chemists," 8th ed. pp. 320-323, Association of Official Agricultural Chemists, Washington, D. C. (1955).

[98] P. J. Porcaro and V. D. Johnston, *Food Technol.* 9, 197 (1955).

To calibrate the column, combine 1 ml of standard solution of coumarin, ethylvanillin, and vanillin containing 1 mg. per ml. in 1:1 chloroform-isooctane. Dilute to 25 ml. with isooctane and add to the top of the chromatographic column. Drive into column with 2-lb. pressure and collect the eluate. Drive 1 ml. of solvent through the column and repeat with 2 1-ml. portions of solvent. Fill the tube to within 1 inch of the top with solvent and drive the solvent through at the rate of 5 ml. per 2-2.5 minutes. Collect 30 5-ml. fractions and read at 270 and 325 mμ against solvent.

Coumarin appears in fractions 6 and 7, reaching a maximum in 8 and 9, ethylvanillin in 11-18, and vanillin in 19-30. If coumarin elutes at fraction 5 or earlier, prepare another column with less water and recalibrate. If coumarin does not appear by fraction 9-10, use more water in the silicic acid.

Pipet a 2-ml. aliquot of the sample in chloroform-isooctane into the column and drive through with 2-lb. pressure. Collect the eluate. Pipet 1 ml. of solvent and drive into the column. Repeat with two 1-ml. portions of solvent. Fill the tube with solvent and continue as described in calibration starting with ". . . drive solvent through at the rate of 5 ml. . . ." Collect 3 more fractions than necessary as indicated by calibration and determine the absorbances of all fractions at 270 and 325 mμ.

Coumarin is identifiable by its absorbance at 325 mμ being slightly more than one-third of its absorbance at 270 mμ. Vanillin and ethylvanillin absorb very little at 325 mμ but also have maxima at about 270 mμ of all fractions containing coumarin and calculate coumarin as follows:

$$c = 12.5 \times \Sigma A \times 1.07/a$$

where ΣA is the sum of absorbances at 270 mμ of fractions containing coumarin

a is the absorptivity of coumarin at 270 mμ

c is the concentration of coumarin in grams per 100 ml. of the original sample

12.5 is the dilution factor

1.07 is the factor correcting for loss during determination.

Calculate ethylvanillin and vanillin similarity. If negative absorbances are obtained on fractions not containing the compounds, correct the absorbances on fractions containing the compounds by adding to each absorbance the average of the negative readings.

By p-*nitroaniline.* To prepare the reagent, dissolve 0.7 gram of *p*-nitroaniline in 9 ml. of concentrated hydrochloric acid and dilute to 100 ml. with water. Cool to 3° and add 5 ml. to 5 ml. of 5% sodium nitrite solution at 3°. After 5 minutes at 3°, add 10 ml. more of sodium nitrite solution at 3° and dilute to 100 ml. with ice water. Let stand for 15 minutes and do not use after 24 hours.

Dilute a 5-ml. sample containing up to 10 mg. of coumarin to 80 ml. and add 5 ml. of a filtered lead acetate solution containing 50 grams each of neutral and basic lead acetate per liter. Dilute to 200 ml. with water, mix, and filter. Discard any cloudy filtrate and dissolve 0.2 gram of anhydrous sodium oxalate in the clear filtrate. Let stand for 5 minutes, filter, and discard any cloudy filtrate.

To 5 ml. of the clear filtrate, add 15 ml. of water and 10 ml. of 1% sodium carbonate solution. Heat at 100° for 5 minutes and let cool to room temperature. Add 10 ml. of reagent, dilute to 100 ml. with water, and mix. After 90 minutes, filter, and read at 490 mμ.

7-GERANOXYCOUMARIN

7-Geranoxycoumarin occurs in a major concentration in grapefruit oil. This is in contrast to cold-pressed lemon oil which contains 5,7-dimethoxycoumarin (limettin) and 5-geranoxy-7-methoxycoumarin.[99] Therefore to detect grapefruit oil in lemon oil chromatograph to obtain a mixture of 5-geranoxy-7-methoxycoumarin and 7-geranoxycoumarin. Both migrate at the same rate on silicic-acid-covered strips. On saponification the phenols differ in their fluorescence so that 0.5% of grapefruit oil in lemon oil is detectable.

For preparation of 100 chromatostrips slurry 57 grams of reagent-grade silicic acid, 3 grams of starch, 0.5 gram of zinc-cadmium sulfide, and 0.5 gram of zinc silicate in 100 ml. of water. Coat 13 × 136 mm. glass strips by flowing the slurry down the inclined strip with gentle tapping of the end to get even spreading, or apply a 0.02-inch coating in a trough prepared for the purpose. After air-drying for a few minutes, dry in a forced-draft oven for 30 minutes. Store over caustic pellets.

For use, first prewash to remove traces of organic matter by putting in stoppered tubes with 1 ml. of petroleum ether until this reaches the top of the strip. Place with the top down and wash with 95% ethanol fed by heavy filter paper. This washing by capillary descent requires 3-4 hours. Activate for use by 6 minutes in a forced-draft oven at 85°.

Apply 0.5 ml. of the oil sample about 2 cm. from the upper end of a chromatostrip. Suspend in a tube with a side-arm reservoir for downward development, holding it with a wire clip through the cork. Fold a band of heavy filter paper over the end of the chromatostrip under the clip extending into the reservoir to serve as a wick to feed solvent to the top of the chromatostrip. Fill the side-arm reservoir with 1:3 diethyl ether-hexane.

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[99] W. L. Stanley and S. H. Vannier, *J. Am. Chem. Soc.* 79, 3488 (1957).

When the first major blue fluorescent spot as shown by long-wave ultraviolet light is near the bottom end, remove and air dry.

Scrape off the silicic acid from the spot and add 2.5 ml. of 1:1 hydrochloric acid. Heat at 100° for 15 minutes and cool. Extract with three 1-ml. portions of chloroform and evaporate the chloroform extracts. Take up the residue in 75% ethanol and dilute to 50 ml. To a 5-ml. aliquot add 2 ml. of 5% potassium hydroxide solution in 75% ethanol and dilute to 10 ml. with 75% ethanol. Read fluorescently at 366 mμ against a reagent blank.

3-(1-PHENYLPROPYL)-4-HYDROXYCOUMARIN

The reaction of 3-(1-phenylpropyl)-4-hydroxycoumarin with diazotized 4-nitroaniline is appropriate for its determination in the absence of other phenolic bodies.[100]

Procedure—As reagent dissolve 1.5 grams of *p*-nitroaniline in 400 ml. of 1:9 hydrochloric acid and dilute to 500 ml. For use cool 25 ml. of this solution below 10° and add 0.75 ml. of a 10% solution of sodium nitrite.

Dissolve a sample containing 3 mg. of 3-(1-phenylpropyl)-4-hydroxycoumarin by heating for 10 minutes at 100° with 12.5 ml. of 10% ammonium acetate solution. Cool and dilute to 25 ml. with the ammonium acetate solution. Filter, add 2 ml. of a freshly prepared diazotized 4-nitroaniline solution to 5 ml. of the filtrate and let stand for 20 minutes at room temperature. Acidify with 1 ml. of concentrated hydrochloric acid. Extract the azo dye with 10 ml. of benzene and dry the benzene with sodium sulfate. Read at 463 mμ. The result is accurate to 5%.

6-METHOXY-7-HYDROXYCOUMARIN, SCOPOLETIN

After separation from interferences by repeated paper chromatography, scopoletin is eluted with 50% ethanol and read at 344 mμ.[101] The procedure described for determination of scopoletin in cigarette smoke assumes that all of the scopoletin found in mainstream smoke from cigarettes is derived from the free scopoletin in the tobacco. Beer's law is followed for 0.01-0.1 mg.

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[100] J. Bednář, *Ceskolov. farm.* 5, 26-7 (1956).

[101] C. H. Yang, Y. Nakagawa, and S. Wender, *Anal. Chem.* 30, 2041-4 (1958).

Procedure—*Cigarette smoke.* Assemble a standard smoking apparatus, using three Kjeldahl flasks in series to trap the smoke. Equip the first 300-ml. flask with a spiral tube connected to the cigarette holder and reaching to the bottom of the flask. Attach a small glass stopper to the end of the side arm so that the stopper could be moved during the smoking procedure and the contents of the spiral tube washed down. Use straight glass tubes for the second and third 100-ml. flasks.

Add 25-ml., 10-ml., and 10-ml. portions of a 1:1 mixture of anhydrous acetone and absolute ethanol to the three receiving flasks and lower into large evacuating flasks containing dry ice-acetone at approximately −77° for at least 30 minutes prior to smoking. Keep the flasks in the cold baths during smoking. Maintain the puff duration at 2 seconds and the puff interval at 60 seconds, smoking regular size cigarettes for 10 puffs with approximately 2 cm. remaining and king size cigarettes for 12 puffs with approximately 3 cm. remaining.

Weigh 10 cigarettes and smoke in the apparatus. Remove the trap system, still connected in series from the evacuating flasks, and let stand until the temperature of the system reaches room temperature. Combine the solvent in the traps and the acetone-absolute ethanol washings and dilute to 100 ml.

Streak three 10-ml. aliquots directly with a 1-ml. micropipet on three chromatographic papers along a 17-cm. line drawn across the paper about 8 cm. from the top, leaving a 1-cm. unstreaked blank at each end of the line. Develop in 15% acetic acid solution. Air-dry the paper and cut off the scopoletin zone, about R_f 0.47, as seen under ultraviolet light at 366 mμ; including an additional 2 cm. from the top and bottom of the zone. Sew the scopoletin zone as tightly and closely as possible to new sheets of chromatographic paper about 8 to 12 cm. from the top of the new sheets. Cut off the portion of paper remaining in back of the scopoletin zone. Develop in a 6:1:2 mixture of *n*-butanol, acetic acid, and water. Again cut off the scopoletin zone, R_f about 0.82, and sew to new paper chromatography sheets as before. Develop the new sheets in 15% acetic acid solution. As soon as the blue fluorescing zone which contains scopoletin moves about 10 cm. beyond the position where the two papers have been sewn together, remove from the developing solution and air-dry. Complete the separation in a 2:3:5 solution of nitromethane, benzene, and water. Cut off the scopoletin zone, sew to new sheets, and develop in 15% acetic acid solution until the blue zone moves halfway down the paper.

To bring the scopoletin into a narrow zone for better elution, cut out the scopoletin area, sew to a new sheet of chromatographic paper and develop in a 6:1:2 mixture of *n*-butanol, acetic acid, and water. Cut out the narrow scopoletin zone and sew one piece of chromatographic paper 9 to 11 cm. long to one narrow end of the scopoletin zone and another piece of paper of the same width but 6 to 7 cm. long to the other narrow end. Cut the shorter addition to a narrow point so that the scopoletin could be concentrated down the point into a container. Put the first addition into a solvent-containing trough in the elution chamber. Elute scopoletin in an elution chamber with 50% ethanol solution to an eluate volume of 8 ml. Dilute to 10 ml. and read at 344 mμ.

Cigarette tobacco. Remove the paper and any filter from 4 cigarettes and without drying, grind the tobacco to pass through a 40-mesh screen. Weigh a 2-gram sample and extract in a Soxhlet extractor using 250 ml. of 85% isopropanol for approximately 3 hours at 100°.

Repeat the extraction and reduce the combined extracts to 150 ml. *in vacuo.* Dilute to 200 ml. with 85% isopropanol. Streak three 15-ml. aliquots separately on three paper chromatograms. Develop in *n*-butanol-acetic acid-water solution and air dry. Cut off the bottom blue fluorescing area containing scopoletin and sew upside down to the end of a new sheet of chromatographic paper so that the scopoletin zone faces the bottom of the sheet. Proceed as for smoke absorber contents.

6-ETHOXY-1,2-DIHYDRO-2,2,4-TRIMETHYLQUINOLINE

The fluorescence of 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline measured in isooctane is the basis for determination of this antioxidant in the presence of impurities. In visible light the compound fluoresces and in ultraviolet light shows a very strong blue fluorescence.[102]

Attempts to separate interfering impurities by chromatography on alumina or magnesia have been unsuccessful. Since 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline is basic, it is extracted quantitatively with dilute sulfuric acid and the fluorescence of the extract is determined before and after treatment with the acid. In certain samples, such as eggs, where the sulfuric acid reacts with components of the sample to produce

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[103] E. M. Bickoff, J. Guggolz, A. L. Livingston, and C. R. Thompson, *Ibid.* 28, 376-8 (1956).

additional fluorescing material, potassium permanganate is used to quench the fluorescence of the quinoline and the sample assayed before and after permanganate treatment.

Beer's law is followed for 0.00001-0.0001 mg. per ml. of the antioxidant. Using quinine sulfate as a fluorometric standard, 0.00006 mg. per ml. in 1:110 hydrochloric acid has a fluorescence equivalent to 0.0001 mg. per ml. of 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline.

Samples—*Tissue.* Blend a 2-gram sample with 50 ml. of acetone. Filter and dilute a 10-20 ml. aliquot with 100 ml. of isooctane. Remove the acetone by washing with water since acetone partially quenches the fluorescence of 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline. Dry the extract over sodium sulfate. Determine the fluorescence using the potassium permanganate technic.

Egg yolk. Since no detectable amount of 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline is found in egg white, wash the sample free from egg white with water. Grind a 2-gram sample with 10 grams of anhydrous sodium sulfate until uniform. Let stand for 1 hour in a desiccator and blend a 4-gram sample with 50 ml. of isooctane for 1 minute. Centrifuge to clarify and determine the fluorescence by the potassium permanganate technic.

Butter. Dissolve a 10-gram sample in 250 ml. of isooctane and shake with 20 grams of sodium sulfate. Centrifuge to clarify and determine fluorescence by the potassium permanganate technic.

Alfalfa meal. Shake a 1-gram finely ground sample for 5 minutes with 30 ml. of diethyl ether and filter. Determine fluorescence in the filtrates by the sulfuric acid technic.

Procedure—*By potassium permanganate.* Determine the total fluorescence at 480 mμ or 435 mμ, making a dilution of the isooctane extract if necessary. Shake 15 ml. of the original filtrate or of the dilution, if one was made, with 10 ml. of 0.04% potassium permanganate solution for 1 minute and decant off the potassium permanganate solution. Dry the isooctane over sodium sulfate and read. Subtract this latter reading from the original reading to obtain the fluorescence attributable to the test substance.

By sulfuric acid. Shake a 25-ml. aliquot of the filtrate of a sample in diethyl ether with 2 portions of 1:69 sulfuric acid and make the extract strongly basic with solid sodium hydroxide. Re-extract with two 25-ml. portions of diethyl ether. Dry the ether extract over anhydrous sodium sulfate and determine the fluorescence directly at 480 mμ or 435 mμ, diluting with ether if necessary.

Gossypol

While the reaction of gossypol with aniline remains the method of determination, the technics have undergone substantial change. The American Oil Chemists' Society Tentative Method Ba 8-55 [103] determines total gossypol and gossypol-like pigments released from cottonseed products under the conditions of the test. Maximum precision is given with samples containing about 0.1 mg. of gossypol.

Up to 0.22 mg. of free gossypol can be determined in mixed feeds by the reaction with phloroglucinol in strong acid at room temperature followed by an extraction with butanone-water azeotrope containing aniline.[104] Free gossypol refers to that portion of the gossypol or gossypol derivatives which may be toxic to animals, or the amount of gossypol brought into solution by a specified solvent under specified conditions. By carrying out the procedure at room temperature interference by reactive substances brought in by the solvent is minimized.

Glucose and other reducing sugars do not interfere. The gossypol reacts preferentially with the aniline in the extracting solvent and thus competing reactions with soluble feed substances are avoided. Feeds which have been treated with aniline can not be determined by this method because dianilinogossypol would be determined as free gossypol. Best results are obtained in the range of 0.02-0.1 mg. per 25 ml. of solution.

A method that can be used with cottonseed products including meals that contain acidulated cottonseed-oil foods or have been aniline treated [105] softens the sample with 72% ethanol before heating with aniline and extracting the dianilinogossypol with chloroform.

Samples—*Cottonseed.* Grind hull-free kernels in a Wiley mill with a 1-mm. screen. Use a sample containing about 1 mg. of gossypol for determination by the AOCS method using aniline.

Cottonseed meal. Grind in a Wiley mill with a 1-mm. screen and use a 1-gram sample.

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[103] "Official and Tentative Methods of the American Oil Chemists' Society," second Ed. including Revisions to 1958.

[104] R. W. Storherr and K. T. Holley, *J. Agr. Food Chem.* **2**, 745-7 (1954).

[105] F. H. Smith, *J. Am. Oil Chemists' Soc.* **35**, 261-5 (1958).

Cottonseed meal or cottonseed meat. Treat sample ground to 40 mesh with 2 ml. of 72% ethanol. Let stand for 10 minutes and determine by aniline using the chloroform-extraction procedure.

Cottonseed meal and feedstuffs. Weigh 0.2 to 2 grams of cottonseed meal or 5-10 gram samples of mixed feed, ground to pass a 1-mm. screen. Add 50 ml. of a solution of 5 ml. of redistilled aniline in one liter of 2-butanone-water azeotrope. Shake meals for one hour and feeds for two hours. Filter and determine gossypol by the phloroglucinol method within one hour.

Cottonseed slab and sized cake. Use the AOCS procedure on sample ground through 1-mm. screen.

Procedure—*By aniline, AOCS procedure.* To a sample containing about 1 mg. of gossypol add 25 ml. of a solution of 12.60 grams of oxalic acid dihydrate in one liter of 2-butanone-water azeotrope. Heat the sample and a blank at 75° for 6-7 hours. Cool and add 25 ml. of 70% aqueous acetone. Pipet 5 ml. of an aqueous 13.673% solution of barium acetate hydrate into each flask. Let stand 10 minutes for barium oxalate to precipitate and filter, discarding the first portion of the filtrate.

Pipet out duplicate 10-ml. portions of the sample filtrate and the blank filtrate. Dilute one of each to 25 ml. with 80% aqueous isopropanol. To the other duplicate aliquots of sample and blank filtrates add 2 ml. of redistilled aniline and heat in a boiling water bath for 30 minutes. Remove from the bath and add enough 80% isopropanol to dissolve the aniline. Cool and make to 25 ml. with 80% isopropanol.

Determine the optical density at 442 mμ of the sample blank against the solvent blank. Determine the optical density of the sample against the reagent blank. Subtract the former from the latter to give the corrected optical density of the sample aliquot.

By aniline, extracted with chloroform.[106] Add 2 ml. of redistilled aniline to a sample in 72% aqueous ethanol. Mix thoroughly and heat on a water bath for 45 minutes. Add 60 ml. of chloroform and shake for 15 minutes. Filter with suction through a filter crucible prepared with asbestos and Hyflo Super-Cel into a 100-ml. volumetric flask. Wash the filter with chloroform and make to volume with it. Transfer 2 ml. of the chloroform filtrate and dilute to 25-ml. with chloroform. Read against chloroform at 440 mμ.

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[106] F. H. Smith, *J. Am. Oil Chemists' Soc.* **35**, 261-5 (1958).

By phloroglucinol. Filter the 2-butanone-water-azeotrope-aniline extract. Transfer a 2-ml. aliquot. Add 1 ml. of 0.4% phloroglucinol in ethanol and 2 ml. of concentrated hydrochloric acid. Let stand 20 minutes and dilute to 25 ml. with ethanol or isopropanol. Use 1 ml. of ethanol for the blank instead of phloroglucinol reagent. Read against the blank at 550 mμ.

HYDROQUINONE

Aside from photometric methods of reading hydroquinone (Vol. III, p. 129) it is read in methyl methacrylate in the ultraviolet.[107] When reacted with titanium sulfate the maximum absorbance is at 503 mμ, page 93.

Procedure—At concentrations of 0.001-0.0025% read at 300 mμ against pure methyl methacrylate as a blank. For concentrations of more than 0.001% dissolve the sample in anhydrous methanol and read at 295 mμ against an appropriate blank.

5,8-DIMETHOXY-2-METHYL-4′,5′-FURO-6,7-CHROMONE, KHELLIN

Khellin, separated from interferences by passage through an alumina and silica column, is read directly in the ultraviolet[108] (Cf. Vol. III, pp. 174-5). The khellin is weakly adsorbed on the alumina, while the chromogens are strongly adsorbed, thus enabling khellin to be washed down with alcohol. Beer's law is followed in the range of 0.012 to 0.0025 mg. per ml., and up to 0.015 mg. per ml. can be determined with an error of 0.0001 mg. The apparatus should be cleaned with concentrated nitric acid, water, and alcohol since khellin is strongly sorbed onto the glass.

When oxidized to khellin-quinone by nitric acid it is read directly.[109]

Procedure—*Blood.* Add a 2-ml. sample of oxalated or heparinized blood to 20 ml. of 95% ethanol. Mix until fine clumps appear and centrifuge for 5 minutes. Decant the supernatant liquid and evaporate at 100°. Stir the precipitate with 5 ml. of ethanol and centrifuge for 5 minutes. Decant the supernatant liquid into the evaporated extract and concentrate to a volume of 7 ml. Cool to room temperature and pass through a 6-mm. column packed with 0.5 gram of pure silica sand at the bottom and 1 gram of alumina under a pressure of approximately 40 mm. of mercury. Collect the total filtrate and wash the column with ethanol until the eluate measures 12.5 ml. Mix the filtrate and washings. Read at 250 mμ against 95% ethanol. Subtract the reading of a blank prepared from the same blood which has been freed of khellin. If such a blank is not available, subtract 0.300 from the optical density reading, equivalent to 0.002 mg. per ml., of the ethanolic extract.

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[107] T. Takeuchi, M. Furusawa, and Y. Takayama, *J. Chem. Soc. Japan, Ind. Chem. Sect.* 59, 118-20 (1956).

[108] F. G. Soloni and J. F. Marquez, *J. Am. Pharm. Assoc.* 42, 20-3 (1953).

[109] J. Correia Ralha, *Rev. port. farm.* 1, 96-8 (1951).

3,3′,4′,5,7-Pentahydroxyflavanone, Dihydroquerecetin, Taxifolin

Dihydroquerecetin reduced with zinc and acetic acid produces a reddish-purple color upon addition of hydrochloric acid.[110] The color intensity is a function of the reaction temperature, particle size, and purity of the zinc. Zinc particle size of less than 60-mesh is unsuitable. Pure zinc gives the lowest color production, while zinc containing copper gives better color formation because copper catalyzes the reaction.

The leucoanthocyanin-like substances in Douglas fir which also produces a similar color is not extracted. Other 3-hydroxyflavanones give the same color but are not present in large enough quantities in wood samples to interfere.

Procedure—*Douglas fir and western larch wood.* Grind a sample to pass through a 40-mesh screen. Weigh 2 grams of sawdust and add 50 ml. of water. Attach an air condenser and heat at 100° for 1 hour. Decant and filter the extract. Repeat the extraction of the sawdust three times: once with 25 ml. of water and heating for 1 hour and twice with 15 ml. portions of water and heating for 30 minutes. Wash the sawdust and dilute the washings and combined extracts to 100 ml.

Add a 1-ml. aliquot of sample solution containing 0.05-0.5 mg. of dihydroquerecetin to 0.5 gram of 30-40 mesh zinc. Add 2 ml. of acetone and 1 ml. of glacial acetic acid. Stir immediately and heat at 65° for 40 minutes. Decant the reduced solution and wash the zinc twice with a total of 10 ml. of acetone. Mix, add 1 ml. of concentrated hydrochloric acid, and mix thoroughly. Dilute to 25 ml. with acetone and read after 5 minutes and within 15 minutes at 525 mμ. The calibration curve must have been prepared with the same lot of zinc.

9-Hydroxyanthracene, Anthranol

In analysis of rhubarb preparations, the characteristic glucoside, rhaponicin, is first separated by paper chromatography. For the descending technic ethanol-water-butanol is appropriate.[111] Thereafter the anthranol and anthraquinone are determined in alkaline solution.

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[110] G. M. Barton and J. A. F. Gardner, *Anal. Chem.* 30, 279-81 (1958).

Procedure—*Total anthraquinones.* Reflux a 0.05-gram sample for 15 minutes with 7.5 ml. of glacial acetic acid. Add 30 ml. of diethyl ether and reflux for an additional 15 minutes. Filter through cotton reserving both fractions. Reflux the residue with 30 ml. of diethyl ether for 10 minutes and filter through cotton. Combine the filtrates and extract with 15 ml. of 30% sodium hydroxide solution. Separate the ether layer and re-extract with 60 ml. of 5% sodium hydroxide solution in 1:13 ammonium hydroxide. Again separate the ether layer and extract with 10 ml. of 5% sodium hydroxide in 1:13 ammonium hydroxide. Combine the red aqueous phases and dilute to 100 ml. with 5% sodium hydroxide in 1:13 ammonium hydroxide. Read at 530 mμ as the reading for anthraquinone. Heat at 100° for 2 hours and cool. Again read at 530 mμ as the value for anthraquinone and anthranol. The difference is anthranol.

Free nonglucosidal anthraquinones. Extract a sample of 0.1-0.2 gram by boiling with 30 ml. of ether for 30 minutes. Proceed as for total anthraquinones from "Filter through cotton. . . ."

ANTHRALIN, DITHRANOL

Anthralin, 1,8,x-anthranol in glacial acetic acid produces a yellow color with sodium nitrite.[112] The maximum intensity is reached in 2 minutes at 100°. Thereafter the color fades at the rate of 3% per minute at 100°. Salicylic acid, benzoic acid, zinc oxide, and boric acid may be present in the sample. Beer's law is followed.

Procedure—*Ointment.* Extract a sample 3 to 4 times with 20-ml. portions of hot glacial acetic acid. Filter each extract. Turbid filtrates will clear upon dilution. Dilute the combined extracts with glacial acetic acid so that 1 ml. contains about 0.02 mg. of anthralin.

Heat a 5-ml. aliquot in glacial acetic acid containing 0.04-0.2 mg. of anthralin with 1 ml. of 5% sodium nitrite solution for exactly 2 minutes at 100°. Cool rapidly to room temperature and dilute to 25 ml. with glacial acetic acid. Read at 450-500 mμ within 10 minutes against water.

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[111] H. Auterhoff, *Deut. Apoth. Ztg.* **94**, 541-5 (1954); **95**, 644-5 (1955).

[112] P. M. Parikh, D. J. Vadodaria, and S. P. Mukherji, *J. Pharm. and Pharmacol.* **11**, 314-7 (1959).

PHENOLIC RESINS

A specific intense yellow color is developed by the action of nitrous acid on phenolic resins dissolved in the water-insoluble solvent of varnishes and other coatings.[113] The color is believed to be due to the formation of *p*-nitrosophenol, which condenses to form other colored complexes. For high accuracy the composition of the resin must be known. However, relatively few phenols produce oil-soluble resins for coatings, so the relative similarity of color developed with the resins indicates that the method has possibilities for estimating resin content based on average color development of a variety of resins. Although each resin and its corresponding phenol used for coatings absorb identically at 433.5 mμ, a wave length of 425 mμ is chosen for the method since this wave length is available on practically all types of photometric instruments.

Other resins, including epoxy resins which use bisphenol, do not interfere. Phenolic antiskinning agents produce color but since the concentration of these agents is small in resins used for coatings, they do not interfere seriously. To analyze materials of unknown phenolic resins origin such as phenol-modified alkyds, compare the color developed by the unknown to the spectra of a representative resin.

Samples—*Resin, varnish, and enamel vehicles.* Centrifuge to obtain the clear pigment-free vehicle. Then dissolve the sample in *n*-butyl acetate and dilute to a definite volume. For insoluble heat-hardening resins, substitute 1-hexanol as the solvent.

Black or olive-drab samples. Clear vehicle cannot usually be separated by centrifuging alone. Dilute with an equal volume of toluene, add 5-10 grams of dry powdered calcium hydroxide to each 100 ml. of sample, and centrifuge.

Procedure—Dilute an aliquot containing up to 6 mg. of phenolic resin to 40 ml. with solvent. Add 10 ml. of 1:10 sulfuric acid and 2 ml. of freshly prepared 10% sodium nitrite solution. Cover with a vented stopper and heat for 1 hour at 70°, agitating gently several times. Cool to room temperature and take up in water. Allow the aqueous layer to separate and remove. Wash the solvent layer twice with water agitating gently. Filter through solvent-dampened paper and dilute to 50 ml. with solvent. Read at 425 mμ against water.

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[113] M. H. Swann and D. J. Weil, *Anal. Chem.* **28**, 1463-5 (1956).

BISPHENOL EPOXY RESINS

Paraformaldehyde develops a specific blue color with bisphenol-type epoxy resins.[114] The color, due to the etherfied diphenol group, is independent of the molecular weight of the polymer, curing of the resin film, and esterfication with acids. Therefore this procedure is applicable to unmodified epoxy resins, their esters, fatty acid esters, dried films, and silicone blends. Paraformaldehyde is substituted for the formalin used in older methods to control the water content of the sample. The temperature must be carefully controlled.

Improved results are not obtained by allowing the colored complex to stand longer than 2 hours or by adding catalysts such as ferric chloride or by heating to speed the rate of color development. Bisphenols also develop color with the reagent but the color development is accelerated when the bisphenols react to form resins. Fatty acids have a retarding effect. Phenolic resins accelerate the color development although they do not produce color themselves.

Pure epoxy resins used as standards should be pulverized, spread out in shallow dishes, and stored in a desiccator over calcium chloride for several days before use.

Procedure—*Bisphenol-type epoxy resins, their esters, dried films,* and *silicone blends.* Weigh a sample containing up to 11 mg. of epoxy resin, dissolve in methyl ethyl ketone, and dilute to a definite volume. If the solution clouds, substitute dioxane for methyl ethyl ketone. Dry an aliquot, not exceeding 3 ml., and containing exactly 15 mg. of solids at 105°-110° overnight. Cool, add 3 ml. of concentrated sulfuric acid, and stopper with a cork provided with a small calcium chloride drying tube. Heat at 40° for 30 minutes, agitating every 10 minutes.

As reagent vehicle add to 10.0 ml. of water about 20 ml. of concentrated sulfuric acid and cool. Dilute to 100 ml. with concentrated sulfuric acid. For use as reagent dissolve 0.15 gram of paraformaldehyde in 10 ml. of the prepared vehicle. The reagent must be prepared fresh daily.

Add 2 ml. of reagent to the hot sample solution and continue to heat 30 minutes at 40°. Pour while hot into 185 ml. of water with vigorous stirring. Dilute to 200 ml. Mix and let stand for 2 hours from the time of pouring into the water. Adjust the volume again to 200 ml. and read at 650 mμ. If readings indicate less than 5 mg. of epoxy resin in the original aliquot, use a portion containing 10 mg. of sample and add exactly 5 mg.

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[114] M. H. Swann and G. G. Esposito, *Ibid.* 28, 1006-7 (1956).

of known epoxy resin before oven-drying the sample, making a correction for the added resin.

Lignin

The content of this complex organic structure can be approximated from the content of phenolic hydroxyl groups.[115] The shift in the maximum when read in neutral and in alkaline solution is best read at 335 mμ. This determines only nonconjugated phenols. Lignin can also be looked at as a polymerized coniferyl alcohol, and low concentrations in pulp determined by reaction with phosphoric acid.[116]

As applied to phenols in lignin the absorbance of the alkaline solution is measured directly against that of a neutralized portion of the same solution to produce a "difference curve." [117] The maxima at approximately 250 and 300 mμ of difference curves obtained by subtracting the spectrum of the neutral solution from that of the alkaline solution of lignin preparations are characteristic for the absorption of the phenolate ion of simple substituted aromatic hydroxyl compounds. Alkaline solutions of phenolic compounds in which the hydroxyl group is conjugated through the ring with a carbonyl group have maxima near 250 and 350 and 400 mμ. The 250 mμ is common to both types of compounds, and the 350 maximum of the difference curves is characteristic for phenolic hydroxyl groups without such conjugation. To insure equal sample concentration in the neutral and alkaline solutions, the neutral solution is prepared from the alkaline solution.

Difference spectra for model compounds give $\Delta\Sigma_{\mathrm{max}}$ value of 3850 at 296 mμ for eugenol and of 4380 at 301 mμ for conidendrin. An assumption is made that the molar absorptivity of the anions of the phenolic structures present in the sample will not differ appreciably from those of the model compounds. Thus an average $\Delta\Sigma_{\mathrm{max}}$ value of 4100 is used.

A small number of phenolic hydroxyl groups conjugated with carbonyl groups and the presence of enolic hydroxyl groups in the side chain conjugated with the benzene ring increases the absorption of the alkaline solution in the region of a longer wave length of 350-400 mμ only slightly.

Lignin in spent sulfite liquor is determined with nitrite and acetic acid as nitrosolignin which is converted to the quinoidal salt with ammonium hydroxide to enhance the color.[118] Since even distilled water gives a nitroso color reaction, a reagent blank is determined using the water used to make up the reagents to cancel out the nitroso color of the water. To do this the absorbance of a solution containing an excess of reagents is read against a solution containing the normal amount of reagents. Since the nitroso color forms in both solutions, only the absorbance of the excess reagents is determined.

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[115] Otto Goldschmid, *Ibid.* **26**, 1421-3 (1954).

[116] David E. Bland, *Australian Pulp & Ind. Tech. Assoc. Proc.* **10**, 287-95 (1956).

[117] Otto Goldschmid, *Anal. Chem.* **26**, 1421-3 (1954).

[118] O. Goldschmid and L. F. Maranville, *Ibid.* **31**, 370-4 (1959).

The absorbance is read against a neutral solution and also against an alkaline solution. Although the absorbances differ, when converted to spent sulfite liquor concentration, the two methods should give identical results. In the presence of interferences, such as extracted tannins or phenolic compounds from sawdust or bark, the second method gives lower results than the first upon conversion of absorbance to concentration. Although the influence of the interferences is greatly reduced by this use of the alkaline blank, neither procedure gives correct results in the presence of interferences. The use of the two methods is helpful in determining the presence of such interferences, however, since a lower value obtained with the alkaline method is an indication of such interferences. Another source of error is turbidity. The sample should be allowed to settle out rather than filtered since lignin sulfonates may be sorbed on the filter.

For determining lignin in high-purity pulps react a 15-gram sample with 100 ml. of 13% nitric acid for 45 minutes at 40°. Filtered and cooled, the filtrate is an arbitrary measure. An absorption at 261 mμ is due to the benzene ring and that at 330-338 mμ to the phenolic group.[119]

Procedure—*Lignin phenolic hydroxyls by difference reading.* Prepare a buffer for pH 12 by dissolving 12.4 grams of boric acid in 0.4% sodium hydroxide solution; dilute to 2 liters with that solvent. Prepare a buffer for pH 6 by mixing 495 ml. of 2.73% potassium dihydrogen phosphate dodecahydrate solution with 113 ml. of 0.4% sodium hydroxide solution and diluting to 2 liters with water.

Dissolve a 0.1-0.3 gram sample in 100 ml. of the buffer solution for pH 12. Dilute a 2-ml. aliquot of this to 50 ml. with the buffer solution for pH 12. To another 2-ml. aliquot, add 2 ml. of 1:350 sulfuric acid and dilute to 50 ml. with the buffer solution for pH 6. Determine the difference spectrum by reading the alkaline solution against the neutralized solution at 280-400 mμ taking readings 2-3 mμ apart in the region of the maximum. Plot the difference spectra of the sample in terms of absorptivity in liters per gram centimeter obtained by dividing the absorbance readings by the concentration of the diluted solutions in grams per liter. Calculate the phenolic hydrovyl group content of the sample using the $\Delta_{a\ \max}$ value of the sample and an average $\Delta_{\Sigma\ \max}$ value for the model compounds, eugenol and corridendrin, as follows:

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[119] Richard Bartunek, *Tappi* 42, 553-6 (1959).

$$\text{Moles of phenolic hydroxyl per gram} = \Delta_{a\,\max}/4100$$

or

$$\%\ \text{phenolic hydroxyl} = \Delta_{a\,\max} \times 17/41.$$

Paper pulp. Add a 5-mg. sample to 5 ml. of concentrated technical grade phosphoric acid. Mix and read after 1 hour at 300 mμ. Calibration is necessarily in terms of the gravimetric method for lignin.

By nitrite. Spent sulfite liquor. Allow a 500-600 ml. sample of spent sulfite liquor to settle overnight. Complete the analysis within 24 hours after collecting the sample. Decant a 450-ml. portion and stir to ensure uniformity. Divide the sample into three 150-ml. portions. To the first add 3 ml. of 10% sodium nitrite solution and 3 ml. of 1:9 acetic acid. Stir, let stand for 15 minutes, and add 6 ml. of 1:9 ammonium hydroxide. Stir. This is the alkaline sample. To the second portion which serves as the alkaline blank, add 3 ml. of 1:9 acetic acid and 6 ml. of 1:9 ammonium hydroxide. Use the third portion as a neutral blank.

Read the neutral blank against the reagent-treated sample at 430 mμ. Compare with the appropriate calibration curve. Then read the alkaline blank against the reagent-treated sample at 430 mμ and compare with an appropriate curve. The two determinations should give the same result.

TANNINS

The compounds called tannins have generally two oxygenated aromatic rings joined by a three-carbon chain of varying oxygen substitution. Phenolic properties predominate although they are also complex acids. They are read directly in the ultraviolet in acidified methanol after extraction from the sample with ethyl acetate.[120] Some materials require a preliminary extraction with a nonpolar solvent such as isooctane. The tannin content is calculated from the extinction coefficient of the tannins present. If this value is not known, it is determined on the sample. Acidification to pH 2 is necessary to ensure complete extraction with ethyl acetate of the undissociated tannin molecules. Acetic acid interferes.

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[120] L. F. Maranville and O. Goldschmid, *Anal. Chem.* 26, 1423-7 (1954); J. L. Owades, G. Rubin, and M. W. Brenner, *J. Agr. and Food Chem.* 6, 44-46 (1958); Cf. Jirí Soudek, *Sborník Ceskoslav. akad. Zeměděl. Ved., Rosttinná Výroba* 3, 937-43 (1957).

Therefore the dried residue of the extract is heated to 80° to drive of acetic acid and ethyl acetate.

Aliphatic reducing substances, ethanol, commercial corn syrup, and caramelized surgar do not interfere. Isohumulones interfere and are removed by treatment of the sample with isooctane. Beer's law is followed up to 40 mg. of tannin per liter.

Difference spectral curves for water extracts of bark materials show maxima near either 290 mμ or 330 mμ or at both wave lengths, except redwood which is virtually structureless and black gum which has maxima at 280 mμ and 350 mμ. Douglas fir has a maximum near 330 mμ but does not exhibit a maximum at 290 mμ. Difference curves of *d*-catechin catechol, resorcinol, and *b*-conidendrol, which showed maxima near 290 mμ, were averaged as the standard for this method providing the average maximum difference in equivalent absorptivity. $\Delta\epsilon_{\max}$, at 1329 liters per equivalent centimeter. The method is useful for the determination o relatively small differences in phenolic hydroxyl content between differen preparations of the same species. Nonconjugated aliphatic enols have no effect while those conjugated with the aromatic ring exhibit a shif of the maximum in alkaline solutions to much longer wave length than phenolic groups. This spectrophotometric determination is somewhat empirical in nature and is dependent upon the availability of suitable models or standard substances.

The absorbance of tannis when read directly at 280 or 203 mμ is based on the polyphenols present. The reading at 203 mμ is unaffected by enzymatic action or darkening. Beer's law is followed.[121] On oxidation the 280 mμ band lessens and one develops at 250 mμ.[122] The difference between the readings in alkaline and neutral solutions described for estimation o lignins is applicable.[123]

A phosphomolybdate-phosphotungstate reagent (Vol. III, p. 461) is specific for all compounds with an oxyphenol bond. Ascorbic acid also reacts with the reagent and gives high results when present in tannin determination. With fruit tannins there is good agreement between this method and the titration with potassium permanganate and indigo carmine, the differences ranging between 3.6 and −7.3%.[124]

Various tannins are also determined by xanthydrol in strong acetic acid solution with boric acid as a catalyst.[125] The sensitivity of the reaction increases with the number of hydroxyl groups in the compound.

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[121] D. G. Roux, *J. Am. Leather Chemists Assoc.* **52,** 319-29 (1957).

[122] C. Yoshimura and K. Ono, *Nippon Kagaku Zasshi* **78,** 320-3 (1957).

[123] L. F. Maranville and O. Goldschmid, *Anal. Chem.* **26,** 1423-7 (1954); Cf. Y Nayndamma, A. Nagisiromanzi, and D. Ramaswamy, *Bull. Research Inst. Madras* 2 285-90 (1956).

[124] C. J. B. Smit, M. A. Joslyn, and A. Lukton, *Anal. Chem.* **27,** 1159-62 (1955).

Samples—*Water extracts.* Grind barks of western hemlock, black gum, Sitka spruce, southern pine, Douglas fir, western red alder, lodgepole pine, white fir, western red cedar, and redwood in a hammer mill and extract with water in a steam-jacketed kettle at 97° under atmospheric pressure. Collect the extract and first wash as 2 fractions, and pass through a 325-mesh screen.

Solvent extracts. Extract bark of western hemlock with acetone. Evaporate the acetone and dissolve the residue in water. Filter and extract the filtrate with ethyl acetate. Evaporate the solvent and take up the residue in water.

Quebracho. Prepare a suitable aqueous solution of solid quebracho or dilute an aqueous extract with water.

Purified aqueous extract. As a control between tannins and non-tannins treat the aqueous extract of western hemlock with standard hide powder to remove the tannins.

Procedure—*Direct reading. Hops.* Homogenize a 20-gram dried sample with benzene in a blender and extract by decanting through filter paper. Wash with approximately 3.5 liters of benzene, or to the disappearance of color. Extract similarly with 2.5 liters of ethyl ether. Air-dry the sample and extract with 700 ml. of boiling water in 5 portions. Concentrate the water extracts to 100 ml. by vacuum distillation and acidify with 1:10 hydrochloric acid to pH 2. Extract the acidified solution with three 100-ml. portions of ethyl acetate and concentrate the ethyl acetate extract to exactly 200 ml. Dry an aliquot, dissolve in methanol, and read at 270 mμ or 203 mμ.

Beer. Acidify a 3-ml. degassed sample with 1 ml. of 1:10 hydrochloric acid and dilute to 25 ml. with water. Add 25 ml. of isooctane, shake vigorously for 30 seconds, and let stand for 5 minutes. Shake 10 ml. of the aqueous phase with 15 ml. of ethyl acetate. Allow the layers to separate and repeat the ethyl acetate extraction twice. Dry the combined ethyl acetate extracts over sodium sulfate and evaporate the solvent. Dry at

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[125] Earl Taubock, *Mitt. Versuchsanstalt Garungsgewerbe u. Insts. angew. Mikrobiol. Hochschule Bodenkult* **3**, 142-3 (1949).

80° for 30 minutes as complete drying is essential for removal of traces of acetic acid and ethyl acetate. Take up with three 2-ml. portions of methanol and add to 0.5 ml. of 1:10 hydrochloric acid. Dilute to 10 ml. with methanol and read at 270 mμ or 203 mμ.

Tea. Extract a 2-gram sample or an infusion of tea leaves or tea solubles with six 50-ml portions of boiling methanol. Concentrate the combined extracts to 80 ml. and add to 400 ml. of chloroform. Vacuum filter and wash with 100 ml. of chloroform, sucking the precipitate almost dry. Dissolve the precipitate in 50 ml. of water and boil off the remaining traces of chloroform. Adjust the pH to 2 with 1:10 hydrochloric acid and extract with three portions of ethyl acetate. Follow the procedure for hops starting at ". . . concentrate the ethyl acetate extract. . . ."

Alternatively, dissolve a 100-mg. soluble sample in 100 ml. of water. Acidify a 2-ml. portion with 1 ml. of 1:10 hydrochloric acid and dilute to 25 ml. with water. Extract a 10-ml. aliquot with ethyl acetate and follow the procedure for beer, starting with "Allow the layers to separate. . . ."

Polyphenolic tannin-like materials by difference reading. As used here polyphenolic refers to materials with more than one phenolic group per aromatic ring.

Prepare a buffer for pH 10 by mixing 43.9 ml. of 0.8% sodium hydroxide solution and 50 ml. of a solution containing 1.24% of boric acid and 1.49% of potassium chloride and diluting to 200 ml. with water. Dissolve 50 mg. of sample in a few ml. of ethanol and dilute to 100 ml. with the buffer for pH 10. The solution of some compounds can be prepared directly in the buffer. Record the time of addition as zero. Dilute a 1-40 ml. aliquot depending on the phenol concentration to 50 ml. with the buffer for pH 10. Add a similar aliquot to 2 ml. of 1:30 sulfuric acid to reduce the pH to about 2.5 and dilute to 50 ml. with the buffer for pH 10. Adjust the aliquots if necessary to give an absorbance reading of approximately 0.5 for the absorbance maximum near 290 mμ. Read the alkaline solution against the second containing sulfuric acid immediately at 290 mμ and 330 mμ, recording the time from the zero time. Measure the difference curve from 260 mμ to 360 mμ with points at 2-3 mμ intervals near the maxima where the times are again recorded. Measure again and convert the three maximum absorbances in liters per gram centimeter to absorptivities and plot against elapsed time in minutes to obtain the extrapolated value at zero time for the maximum at both 290 and 330 mμ, $\Delta_{a\ \mathrm{max}}$.

Calculate the phenolic hydroxyl group content of the polyphenolic samples from the values of $\Delta_{a\ \mathrm{max}}$ at 290 and 330 mμ and from the average value of $\Delta_{\Sigma\ \max}$ for the model compounds, catechol and resorcinol, as follows:

$$\text{Moles of phenolic hydroxyl per gram} = \Delta^{290}_{a\ \max}/1329 + \Delta^{330}_{a\ \max}/5705$$

$$\%\ \text{phenolic hydroxyl} = 1.28\Delta^{290}_{a\ \max} + 0.298\Delta^{330}_{a\ \max}.$$

By phosphomolybdate-phosphotungstate. Fruit including both green and ripe persimmon, apple, peach and pear. Blend a 50-gram sample with 230 ml. of 95% ethanol and reflux for 15 minutes. Filter, add 20 ml. of a saturated neutral lead acetate solution, and adjust the pH to 7 with 10% sodium hydroxide solution. Let stand overnight. Centrifuge and wash the precipitate with three small portions of water. Remove the lead by passing through Amberlite IR-120 in the hydrogen form. Filter and concentrate to 50 ml. on a steam bath. Add 50 ml. of water and concentrate again to 50 ml. at 100° to remove all ethanol. Dilute to 100 ml.

To prepare the reagent, reflux 100 grams of sodium tungstate, 20 grams of phosphomolybdic acid, and 50 ml. of 85% phosphoric acid with 750 ml. of water for 2 hours. Cool and dilute to 1 liter. To an aliquot of sample containing up to 1 mg. of tannin, add 75 ml. of water, 5 ml. of reagent, and 10 ml. of a saturated sodium carbonate solution. Dilute to 100 ml. with water and read after 30 minutes at 760 mμ.

Butylated Hydroxyansole

An indirect method for the determination of the antioxidant butylated hydroxyanisole is to oxidize with ferric sulfate and react the resulting ferrous sulfate with ferricyanide.[126] The blue color of the ferrous ferricyanide is measured. A linear relationship exists between increasing quantities of butylated hydroxyanisole and the intensity of the blue color of the ferrous ferricyanide complex. The color is stable for 1 hour.

It is also extracted with methanol and chloroform and read directly in the ultraviolet.[127] The sample should not contain over 100 ppm. butylated hydroxytoluene and tocopherols interfere.

Procedure—*By ferric ion and ferricyanide.* Dilute a fat sample containing 0.004-0.03 mg. of butylated hydroxyanisole to 10 ml. with 72% ethanol. Add 0.5 ml. of 0.2% potassium ferricyanide solution and 0.5 ml. of 0.5% ferric sulfate solution in 1:35 sulfuric acid. Mix and allow the color to develop for 15 minutes in the dark. Read at 660 mμ.

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[126] K. Krishnamurthy and M. Swaminathan, *Current Sci.* (*India*) **25**, 16-7 (1956).

[127] P. V. Hansen, F. L. Kauffman, and L. H. Wiedermann, *J. Am. Oil Chemists' Soc.* **36**, 193-5 (1959).

Direct reading. Melt a 25-gram sample of fat and add 10 ml. of chloroform and 100 ml. of methanol. Shake for a minute and chill to precipitate the fat. Filter and set aside the solvent layer. Repeat the extractio twice with 5 ml. of chloroform and 50 ml. of methanol. Evaporate th combined filtrates to 10 ml. and filter. Wash the filter with cold methano and dilute the filtrate to 25 ml. Read at 270, 290, and 310 mμ. Correct fo dissolved fat by the formula:

$$\text{Corrected } d_{290} = d_{290}\,[(d_{290} - d_{310})/2].$$

CHAPTER 5

QUINONES

ADRENOCHROME appears in this chapter as the 3,3-dihydro-3-hydroxy-*N*-methylindole-5,6-quinone. However it is the product of mild oxidation of epinephrine (adrenaline) and is commonly as intermediate in determination of the latter (page 111).

1,2-Naphthoquinone gives a green color with sulfuric acid under conditions described for determination of menadione with the same reagent. This reaction is also applicable to 1,4-naphthoquinone, page 145. For direct reading in methanol in the presence of naphthalene and phthalic anhydride see page 23. Anthraquinone is read in aqueous alkaline solution as a part of the determination of anthranol, page 132. Podophyllotoxin gives a maximum fluorescence at pH 11 and 325 mμ. Mephenesin reacts similarly at pH 1 and 315 mμ.[1]

BENZANTHRONE AND 2,2′-DIBENZANTHRONYL

In the determination of benzanthrone and 2,2′-dibenzanthronyl, dissolved in concentrated sulfuric acid, a wave length of 374 mμ is chosen for benzanthrone since at this point the ratio of benzanthrone absorption to that of dibenzanthronyl is at a maximum of 1.72. Similarly a wave length of 550 mμ is chosen for dibenzanthronyl since at this point the absorption is almost 5 times as intense as that of benzanthrone.[2] Since neither of these wave lengths are points of maximum absorption all work should be carried out with slit widths of 0.15 and 0.035 mm. at 374 mμ and 550 mμ respectively to eliminate error of slit-width effect. The absorbancies are additive over a range of 0.002-0.01 mg. of both components and Beer's law is followed within this range.

Dibenzanthrone, violanthrone, interferes at both wave lengths but the amount can be determined because it exhibits a pronounced absorption maximum at 850 mμ where neither benzanthrone nor 2,2′-dibenzanthronyl show measurable absorption. Thereafter a correction factor can be applied for low concentrations of dibenzanthrone since 1% is equivalent to 0.005 absorbance unit at a concentration of 0.01 mg. per ml. For each per cent of dibenzanthrone, 0.003 absorbance unit is subtracted from the sample absorbance at 374 mμ and 0.004 unit from the reading at 550 mμ.

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[1] S. Udenfriend, D. E. Duggan, B. M. Vasta, and B. B. Brodie, *J. Pharmacol. Exptl. Therap.* 120, 26-32 (1957).

[2] Kermit B. Whetsel, *Anal. Chem.* 26, 1974-7 (1954).

In all determinations the sulfuric acid reagent should meet the ACS specification since trace amounts of oxides of nitrogen nitrate the dibenzanthronyl to cause erratic results. Error is also caused by background absorption of some samples. Benzanthrone is also read in the presence of anthraquinone, page 149.

Procedure—*Benzanthrone and dibenzanthronyl.* Dissolve a 50-mg. sample in 500 ml. of sulfuric acid and dilute a 10-ml. aliquot to 100 ml. with sulfuric acid. Read at 374 mμ with a slit width of 0.15 mm. against sulfuric acid, and read at 550 mμ with a slit width of 0.035 mm. against sulfuric acid. Determine the cell corrections at 374 mμ and 550 mμ by measuring the absorbance at these wave lengths with the cell filled with sulfuric acid and apply these corrections to the absorbancies obtained with the sample. Calculate as follows:

$$\%\text{ benzanthrone} = 119.8\,A_{374\,\text{m}\mu} - 134.0\,A_{550\,\text{m}\mu}$$

$$\%\text{ dibenzanthronyl} = 231.9\,A_{550\,\text{m}\mu} - 24.5\,A_{374\,\text{m}\mu}.$$

2-Methyl-1,4 Naphthoquinone, Menadione, Vitamin K_3

The 2,4-dinitrophenylhydrazine method (Vol. III, p. 169) is modified to permit the determination of micro quantities of menadione, 2-methyl-1,4-naphthoquinone, in the range of 0.002-0.3 mg.[3] The rate of color formation depends on the concentration of menadione and the period of incubation. The color is stable for 24 hours at an incubation temperature of 35° while colors developed at higher temperatures are more intense but less stable.

Alcoholic solutions of 1,4-naphthoquinones and their 2-methyl derivatives give a red color when heated with concentrated sulfuric acid which changes to a violet color upon cooling and dilution with water.[4] A green color which changes to a rose color upon cooling is formed by a similar reaction between 1,2-naphthoquinones with position 3 unsubstituted.

The treatment of menadione with concentrated hydrochloric acid gives a dark red color which has been formulated as the quinhydrone of 3-chloro-2-methyl-1,4-naphthoquinone and 2-methyl—1,4-naphthoquinone. Beer's law is followed for 0.1-0.25 mg. of menadione.[5] The reaction is also applied with 1% of zinc chloride in concentrated hydrochloric acid with the maximum absorption at 510 mμ after 30 minutes at 100°.[6]

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[3] V. Sathe, J. B. Dave, and C. V. Ramakrishnan, *Nature* 177, 276 (1956); *Anal. Chem.* 29, 155-6 (1957).

[4] Maria Isabel Ardao, *pR* (*Montevideo*) 2, 26C-37C (1952).

[5] J. A. de Almeida Baltazar, *J. Farm* (*Lisbon*) 9, 57-8 (1950); *Rev. port. farm.* 3, No. 1, 1-8 (1953).

Cysteine hydrochloride is another reagent for menadione when made alkaline.[7] Beer's law is followed for samples containing up to 0.1 mg. of vitamin K_3 per ml. The optical density of the color is not changed with the variation of cysteine hydrochloride concentration of 0.075-0.025% and a sodium hydroxide concentration of 0.4-7%. Another reagent for the purpose is dimethylaniline.[8]

Sample—*Pharmaceutical preparations.* Powder a 50-mg. sample and add 20 ml. of ethanol. Heat to 40° for 5 minutes with agitation, filter, and develop a 0.5-ml. aliquot with concentrated hydrochloric acid.

Procedure—*By 2,4-dinitrophenylhydrazine.* Shake 0.1 ml. of sample containing 0.002-0.3 mg. of menadione with 0.5 ml. of ethanol and add 0.1 ml. of a saturated 2,4-dinitrophenylhydrazine solution in 1:5 hydrochloric acid. Incubate at 35° for 10 minutes and cool to room temperature. Add 0.3 ml. of 20% sodium carbonate solution and shake well until the green color appears. Add 3 ml. of amyl alcohol, 1 ml. of ethanol, and 1 ml. of water. Shake thoroughly and let stand for 5 minutes to allow salts to settle. Separate the amyl alcohol layer and read this layer at 635 mμ against a reagent blank.

By concentrated sulfuric acid. Mix equal volumes of concentrated sulfuric acid and the naphthoquinone in solution in 70-80% ethanol at around 0.01 mg. per ml. Heat at 100° for 5-10 minutes, cool, and dilute with 8 volumes of water. Read at 550 mμ for 1,4-naphthoquinones and their 2-methyl derivatives.

By concentrated hydrochloric acid. Treat a 0.1-0.5 ml. sample containing 0.05-0.25 mg. of menadione with 1 ml. of concentrated hydrochloric acid for 5 minutes at 100°. Add 1 ml. of concentrated hydrochloric acid and heat at 100° for 10 minutes. Dilute to 20 ml. with ethanol and read the red color at 520 mμ.

By hydrochloric acid-cysteine. To a 1-ml. sample containing less than 0.1 mg. of vitamin K_3, add 3 ml. of 0.05% cysteine hydrochloride solution and 1 ml. of 4% sodium hydroxide solution. Let stand for 15 minutes and read at 440 mμ.

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[6] F. J. Bandelin and R. E. Pankratz, *Drug Standards* 27, 36-9 (1959).

[7] M. Yamagishi, H. Fujiwara, and S. Shiraki, *Am. Repts. Takeda Research Lab.* 13, 16-9 (1954).

[8] Masaharu Yamagishi, *Ibid.* 13, 20-4 (1954).

5,7,3′,4′-TETRAHYDROXY-3-FLAVANOL, QUERCETIN

Quercetin reacts with aluminum chloride to form a colored comple containing 1 mole of quercetin per mole of aluminum chloride and exhibiting a maximum absorption at 430 mμ.[9] The color forms immediately an the absorbance remains constant for 10 to 90 minutes. A 200-mole exces of aluminum chloride is needed for complete complexing. Each batch o aluminum chloride reagent must be standardized against pure quercetin In some samples interfering flavonoids must be separated by chromatography and a correction applied for the absorption of quercetin by th paper. Phlobaphenes and tannins, which interfere if the complex is rea in alcoholic solution, do not interfere when the complex is read in aluminum chloride solution at pH 4. Dihydroquercetin forms a colorless complex with the reagent and does not interfere. Beer's law is followed fo 0.002-0.015 mg. of quercetin per ml. of reagent. Since the variation fron linearity is slight below 0.002 mg. per ml., low concentration of querceti may be read with only a slight decrease in accuracy.

QUERCETIN

Quercetin and rutin both form yellow complexes with antimony tri chloride (cf. Vol. III, p. 174). The quercetin complex absorbs at 470-50 mμ but the rutin complex does not absorb in this range. Therefore th reaction is used to determine each.[10]

First read the complex in methanol at 470 mμ and calculate the resu as quercetin. Then hydrolyze the sample with 1:99 hydrochloric acid i methanol, neutralize with sodium carbonate, and read at 470 mμ. Th rutin in the sample is the difference between the quercetin content in th unhydrolyzed sample and in that in which the rutin has been hydrolyze to quercetin and rutinose. After chromatographic isolation, quercetin an rutin are determined by reaction with the diazo compounds of *p*-aminobenzoic acid, sulfanilic acid, *o*-, *m*-, and *p*-nitroaniline.[11]

Samples—*Douglas fir bark.* Dry the sample in 1.5-gram portions fo 2 hours at 105° under 28 inches of vacuum. Weigh out a sample containing 0.1-0.13 gram of anhydrous quercetin and take up in 125 ml. of isopropanol. Dissolve by heating to 50° for 10 minutes, swirling gently. Co to 20°, dilute to 250 ml. with water, and mix. Let any insoluble solids settl out for 15 minutes. Develop a 1-ml. aliquot with aluminum chloride.

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[9] L. E. Dowd, *Anal. Chem.* 31, 1184-7 (1959).

[10] D. Gr. Constantinesco and R. Oteleanu, *Farmacia* 4, 244-52 (1956); *Acta Polo Pharm.* 14, 27-32 (1957).

[11] J. Davidek and J. Fragner, *Nahrung* 2, 401-7 (1958).

Onions. Slurry a sample with water in a blender and vacuum-dry the pulp at 65°. Extract quercetin from 10 grams with methanol for 6 hours. Concentrate the methanol to 5 ml. and dilute to 100 ml. with water. Extract 3 times with equal volumes of diethyl ether to separate quercetin from any quercetin glycosides. Evaporate the ether to dryness and extract the residue 3 times with 10-ml. portions of hexane and 3 times with 25-ml. portions of ethylene dichloride to remove fats and oils. Extract 3 times with 10 ml. of water to remove water-soluble material which was extracted with the ether.

Dissolve the remaining residue in 50 ml. of isopropanol. Concentrate 25 ml. to 5 ml. and spot 0.64 ml. on one chromatographic paper and a known amount of pure quercetin on another paper. Irrigate both papers with a 22:78 mixture of isopropanol and water (R_f quercetin 0.06). Extract the quercetin with a 30:70 mixture of ethyl acetate and ethanol and dilute the eluate to 5 ml. Evaporate a 2-ml. portion to dryness and dissolve the residue in a few drops of isopropanol. Develop with aluminum chloride.

Procedure—*By aluminum chloride.* To prepare the reagent, boil 20 liters of water for 10 minutes, cool to 90°, and add 48.28 grams of aluminum chloride hexahydrate. Adjust the pH to 4 with 20-30 grams of potassium acetate crystals, checking the pH in a small portion of solution cooled to room temperature. Let stand for 24 hours and store by sealing with a stopper containing a siphon arrangement and a breathing tube filled with soda lime. The reagent is stable for several weeks.

Dilute a 1-ml. aliquot from the Douglas fir bark sample to 100 ml. with aluminum chloride reagent or dilute the sample from onions to 10 ml. with the reagent. Read at 430 mμ against aluminum chloride after 10 minutes. If the sample has been chromatographed, calculate the amount of quercetin absorbed by the paper and apply a correction to the unknown.

HYPERICIN, HEXADHYDROXY-2,2′-DIMETHYLNAPHTHOANTHRONE OR HEXADHYDROXY-2,2′-DIMETHYLHELIANTHRONE

Hypericin is read directly in pyridine-methanol solution at 591 mμ.[12] Beer's law is not followed.

Procedure—*Herba hyperici.* Dry a 10-50 gram sample at 45°, powder and dry at 50°. Extract a 1-5 gram sample with chloroform in a Soxhlet apparatus and dry the extract at 50°. Extract the residue with 3% pyridine solution in methanol in a Soxhlet apparatus, filter, and dilute to 100 ml. with 3% pyridine solution in methanol. Read at 591 mμ.

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[12] F. Neuwald and U. Hagenstrom, *Arch. Pharm.* **288,** 38-42 (1955).

ADRENOCHROME

3,3-DIHYDRO-3-HYDROXY-N-METHYLINDOLE-5,6-QUINONE

Adrenochrome in the presence of zinc acetate forms a fluorescent compound, adrenolutin, 3,5,6-trihydroxy-*N*-methylindole, which exhibits a linear fluorescent curve for 0.0001-0.001 mg. of adrenochrome.[13] Since adrenochrome, an intermediate stage in the oxidation of epinephrine by a mammalian catechol oxidase in human plasma, has not been isolated from plasma, it is only assumed that this procedure measures adrenochrome.

The fluorescence is not altered between pH 3 and pH 7. Plasma proteins, which have a quenching effect, are precipitated. 2-Iodoadrenochrome, 5,6-dihydroxynorephedrinechrome, and noradrenochrome yield fluorescent substances with zinc salts but the latter two compounds have not been found in blood plasma. Adrenaline, noradrenaline, tryptophan, tryptamine, 5-hydroxytryptophan, serotonin, 3-indoleacetic acid, 5,6-dihydroxy-*N*-methylindole, 3,4-dihydroxyphenylalanine, dopachrome, epinine, epinochrome, and adrenoxyl do not give increased fluorescence after addition of zinc salts in acetone and do not interfere.

Procedure—*Blood.* Mix 5 ml. of heparinized blood and 10 ml. of 10% trichloroacetic acid. Filter. Place 2 ml. of filtrate in Tubes A_1, B_1, S_1, and S_2. Place 5 ml. of water in Tubes A_2 and B_2. Add 0.1 ml. of adrenachrome standard containing 0.002-0.003 mg. to Tubes S_1 and S_2. Add 0.1 ml. of water to the other tubes. Add 2 ml. of 10% trichloroacetic acid to all tubes. Add 1 ml. of 22% zinc acetate solution in 10% aqueous trichloroacetic acid to Tubes A_1, A_2, and S_2. Mix and after 10 minutes read with excitation at 410 mμ and emission at 500 mμ. The tubes may be stored at 4° for 60 minutes with low concentrations, for 15 minutes at high concentrations.

$$(A_1 - B_1) - (A_2 - B_2) = R$$

$$\text{micrograms of adrenochrome per liter} = 3/2 \times R \times \frac{1000}{(S_1 - S_2) - (A_1 - B_1)}.$$

ANTHRAQUINONE

Anthraquinone shows a maxmium absorption at 266 mμ when dissolved in concentrated sulfuric acid. While benzanthrone also absorbs at this wave length, the absorption by anthraquinone is almost 6 times as great. The presence of the latter can therefore be corrected. Benzanthrone under similar conditions has a maximum at 515 mμ. There is no interference by anthraquinone at that wave length.[14] Under other circumstances benzanthrone is read in the same solvent in the presence of 2,2′-dibenzanthronyl, given as a separate method. Small amounts of anthrone interfere little at 266 mμ and not at all at 515 mμ.

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[13] A. N. Payza and M. E. Mahon, *Anal. Chem.* **31**, 1170-4 (1959); **32**, 17-9 (1960).

Beer's law is followed for 1 part of anthraquinone per 100,000 parts of acid and for a similar concentration of benzanthrone. Samples should not stand for long periods of time before reading since the absorption at 515 mμ increases on standing. The sulfuric acid must meet ACS standards since more than a trace of nitric acid interferes. In analysis of anthranol in rhubarb it is necessary to determine total anthraquinones and then apply a difference method, page 132.

Procedure—*Anthraquinone and benzanthrone.* Dissolve a 50-mg. sample in 500 ml. of concentrated sulfuric acid and dilute a 10-ml. aliquot to 100 ml. with sulfuric acid. Read at 266 mμ and 515 mμ against sulfuric acid. Determine the cell correction by measuring the absorbance with sulfuric acid. Using the corrected absorbancies calculate the components as follows:

$$\%\text{ anthraquinone} = 55.07\,A_{266\,\text{m}\mu} - 41.07\,A_{515\,\text{m}\mu}$$

$$\%\text{ benzanthrone} = 241.0\,A_{515\,\text{m}\mu}.$$

1,4-DIHYDROXYANTHRAQUINONE

This intermediate is determined in certified dyes D & C Violet No. 2 and D & C Green No. 6. The reading is directly in the ultraviolet.[15]

Procedure—Suspend 100 mg. of dye in 150 ml. of diethyl ether. Extract with about 25-ml. portions of 0.8% sodium hydroxide solution so long as color is removed. Wash the combined alkaline extracts with 50-ml. portions of ether until the extracts are colorless. Discard the ether extracts and heat the alkaline solution for 1 hour at 100° to remove ether. Cool, dilute to 250 ml., and read at 230 mμ, 255 mμ, and 280 mμ.

% 1,4-dihydroxyanthraquinone

$$= \frac{A_{255}\text{ unknown} - A_{230}/2 - A_{280}/2}{A_{235}\text{ standard} - A_{230}/2 - A_{280}/2} \times \begin{array}{c}\text{concentration of standard}\\ (\text{mg./l.}) \times 20.\end{array}$$

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[14] Kermit B. Whetsel, *Ibid.* **25,** 1334-7 (1953).

[15] L. S. Harrow and K. S. Heine, Jr., *J. Assoc. Offic. Agr. Chemists* **35,** 751-4 (1952).

CHAPTER 6

OXYGEN CYCLES, OXIDES, AND PEROXIDES

IN THE determination of cantharidin, hexahydro-3a,7a-dimethyl-3,7-epoxyisobenzofurane-1,3-dione, *m*-dihydroxybenzene may replace formaldehyde (Vol. III, p. 183). Then the green fluorescence is read at a controlled degree of alkalinity. For determination of ethyl Cellosolve by Sudan III see page 40. Parsley camphor or apiole, 1-allyl-2,5-dimethoxy-3,4-methylenedioxybenzene, is determined by chromotropic acid.[1]

In reading of safrole after steam distillation and extraction with chloroform the strongest absorption is at 287 mμ, see Vol. III, p. 177.[2]

ETHYLENE OXIDE AND 1,2-EPOXIDES

Ethylene oxide has been determined by its brown color with pyridine (Vol. III, p. 500). Periodate oxidizes it to formaldehyde which is determined by sodium chromotropate[3] (Vol. III, p. 259), phenylhydrazine[4] (Vol. III, p. 258), or acetylacetone.[5] For samples of air the ethylene oxide is sorbed on silica gel. Neither acids nor alkalies interfere.

Essentially complete conversion to aldehyde and determination with chromotropate is obtained over the range of 6-136 ppm. of ethylene oxide. If a 20-gram sample is used, the lower limit of detection is 1 ppm. This limit may be expanded by use of a larger sample.

If materials which react with periodate are absent the sample can be used directly. Otherwise ethylene oxide must be separated by evolution from boiling water. Only 86% of the ethylene oxide is so evolved the balance being hydrolyzed to ethylene glycol. A separate calibration curve is therefore required when the evolution procedure is used. And of course other volatile interferences, propylene oxide for example, must be absent With the sodium chromotropate reagent, Beer's law is followed for 0.15-0.75 mg. of ethylene oxide. The colors are stable for 48 hours. Since ethylene glycol reacts similarly to ethylene oxide, ethylene glycol may be substituted as standards.

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[1] E. Bovalini and A. Casini, *Ann. chim.* (*Rome*) 49, 1059-66 (1959).

[2] John B. Wilson, *J. Assoc. Offic. Agr. Chemists* 42, 696-8 (1959).

[3] F. E. Critchfield and J. B. Johnson, *Anal. Chem.* 29, 797-800 (1957).

[4] M. E. Brokke, U. Kiigimage, and L. C. Terriere, *129th Meeting of The American Chemical Society* (April, 1956).

[5] J. C. Gage, *Analyst* 82, 587-9 (1957).

A detector to determine ethylene oxide in air to ±5% is 2 mm. in diameter filled with 0.2 gram of silica gel on which potassium dichromate acidified with sulfuric acid has been sorbed and dried.[6] The changed color-length of the detecting column from orange-yellow to dark yellow green is measured after 100 ml. of gas has been passed through at the rate of 1 ml. per second, or by measuring the volume of gas required to change the color up to a certain length of the detecting column. Up to 4% by volume of ethylene oxide can be estimated by the former method or from 0.003-0.5% by the latter. Alcohols, aldehydes, diethyl ether, sulfur dioxide, hydrogen sulfide, acetone, benzene, aniline, petroleum, carbon disulfide, nitrous oxide, and hydrochloric acid must be absent.

Procedure—*By sodium chromotropate. Interference present.* The evolution apparatus is shown in Figure 7. To prevent reaction of ethylene oxide with lubricants, use silicone grease in all joints.

Place 25 ml. of 3.5% hydroxylamine hydrochloride solution in scrubber A. This is to remove interfering aldehydes from the air. Add 200 ml. of water to flask C, using a larger flask if the samples foam considerably. Add a sample containing not more than 0.7 mg. of ethylene oxide to the flask, stopper, and thoroughly mix. Introduce 20 ml. of water at 5° to absorber E and connect the scrubber to the reflux condenser by means of the ball and socket joint. Immerse the scrubber in flask F containing an ice-water slurry. Adjust the air flow to 10 ml. per minute, connect scrubber A to ebullator B in the distillation flask by means of rubber tubing, and turn on the condenser water which should not be above 10°. Start heating and reflux for 30 minutes. Stop heating, remove flask F, and rinse the drain tube of the absorber with water.

Interferences absent. Take a sample containing not more than 0.7 mg. of ethylene oxide or the contents of absorber E. In either case add the sample to a heat-resistant pressure bottle. Rinse in with a minimum amount of water. Prepare a blank by adding water to a second pressure bottle to the same level as the sample. Add 1 ml. of 1:70 sulfuric acid to each pressure bottle and stopper, protecting the rubber stoppers with a polyethylene film. Heat in fabric bags for 1 hour at 98 ± 2° to convert to ethylene glycol. Cool to room temperature, loosen the bags, carefully un-

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[6] T. Kitagawa and Y. Kobayashi, *J. Chem. Soc. Japan, Ind. Chem. Sect.* **58**, 514-7 (1955).

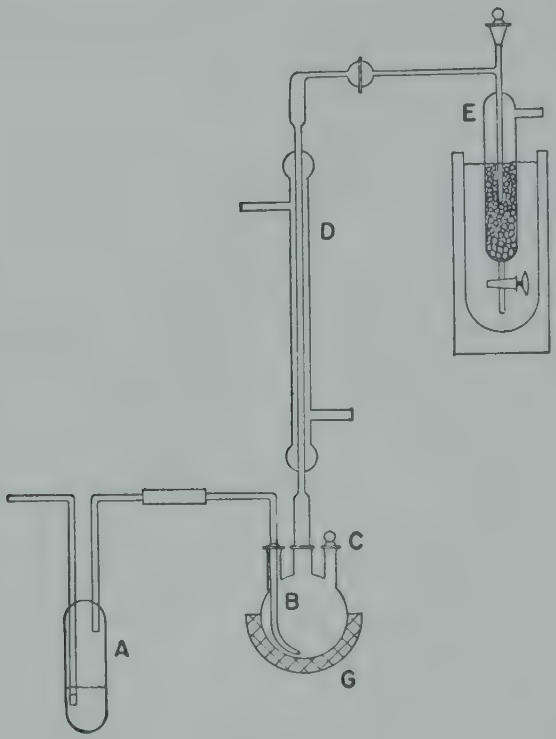

FIG. 7

Apparatus for evolution of ethylene oxide

A. Scrubber, 250 ml., containing hydroxylamine hydrochloride and triethanolamine. B. Ebullator with capillary tip. C. Distillation flask, 1 liter. D. Wate condenser. E. Scrubber, 50 ml., containing glass beads with capillary tip ebullator F. Dewar flask containing ice-water slurry. G. Glas-Col heating mantle.

cap to release the pressure, and remove the bags. Add 1 ml. of 2% sodiun hydroxide solution, stopper, and mix. Add 2 ml. of 2.1% sodium periodat solution, mix, and let stand for 15 minutes to permit conversion to formaldehyde. To reduce excess periodate, add 2 ml. of 5.5% sodium sulfite solution and dilute to 100 ml. with water. Stopper and mix.

To a 10-ml. aliquot of the sample and blank add 0.05 gram of sodiu chromotropate and shake to dissolve. Dilute to 50 ml. with concentrate sulfuric acid. Bubble nitrogen through vigorously for 10 minutes and le cool to room temperature. Read the sample against the blank at 570 m

By acetylacetone. In air. Prepare a piece of glass tubing having a internal diameter of 6 mm. and about 180 mm. long by making a serie of indentations about 40 mm. from one end to hold cotton-wool. Add grams of 40-60 mesh silica gel, plug with another wad of cotton-wool, an close with caps or stoppers until needed. Connect the tube to a water aspirator or another suction device and draw the air sample containing up to 0.04 mg. of ethylene oxide through the tube at the rate of 0.5 liter per minute.

Empty the contents of the tube into 10 ml. of water. Add 1 ml. of 3.84% periodic acid solution and heat at 100° for 40 minutes to oxidize the ethylene oxide to formaldehyde. Add 2 ml. of 6.5% sodium arsenite solution and 2 ml. of acetylacetone reagent containing 25 grams of ammonium acetate, 3 ml. of glacial acetic acid, and 0.2 ml. of redistilled acetylacetone in 100 ml. of water. Stopper and heat for 3 minutes at 100°. Decant the clear liquid and filter if turbid. Read at 412 $m\mu$ against a reagent blank.

Ethylene Oxide Addition Products

A red color forms upon addition of sulfuric acid to the type of non-ionic detergents formed by addition of ethylene oxide. The addition of phosphomolybdic acid produces a more sensitive violet to rose-pink color which is specific for these non-ionic detergents.[7] The method will detect 0.01 mg.

An indirect method for these non-ionic detergents is based on the determination of molybdenum in the phosphomolybdate complex precipitate of the detergent. The molybdenum is developed with thiocyanate and stannous chloride, and may be read in the aqueous solution or extracted with butyl acetate. Reading in the aqueous phase is preferable as difficulties may be encountered in the extraction. The slight turbidity which appears in the solution does not interfere with the determination. This determination of molybdenum is more sensitive than the sulfuric acid method.

In both methods the salts of ethylene diamine tetraacetic acid do not interfere, while carboxymethylcellulose and alkyl sulfates cause low readings probably because of the protective action of the phosphomolybdate precipitate which results in the incomplete separation on centrifuging. A slight increase in the result is caused by alkylaryl sulfonates.

Procedure—*By sulfuric and phosphomolybdic acids.* Dilute a sample containing 0.01-0.5 mg. of non-ionic detergent to 5-10 ml. Add 3 drops of 1:4 hydrochloric acid, 2 drops of 10% barium chloride solution, and 2 drops of 10% phosphomolybdic acid solution. Stir well and centrifuge the precipitate. Washing is not necessary as the reagents give no color. Dissolve the well-drained precipitate in concentrated sulfuric acid with stirring and warming if necessary. Dilute to 4 ml. with concentrated sulfuric acid and read after 40 minutes at 520 mμ.

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[7] D. G. Stevenson, *Analyst* **79**, 504-7 (1954).

By thiocyanate and stannous chloride. To prepare the reagent, dissolve 35 grams of stannous chloride in 10 ml. of concentrated hydrochloric acid and dilute to 100 ml. with water. Add metallic tin to keep the reagent in a reduced state.

Dilute a sample containing 0.01-0.5 mg. of non-ionic detergent to 5-10 ml. Add 3 drops of 1:4 hydrochloric acid, 2 drops of 10% barium chloride solution, and 2 drops of 10% phosphomolybdic acid solution. Stir well, centrifuge, and wash the precipitate with 5 ml. of water. Centrifuge, decant, and dissolve the precipitate in 1-2 ml. of concentrated sulfuric acid. Dilute to 6 ml. with water. Add 1 ml. of 5% ammonium thiocyanate solution and 0.5 ml. of stannous chloride solution. Dilute to 10 ml., mix well, and centrifuge if necessary. Read after 20 minutes at 470 mμ. If extraction is required, extract the molybdenum thiocyanate with butyl acetate previously saturated with ammonium thiocyanate and stannous chloride..

TERT-BUTYL PEROXIDE

Vanillin in sulfuric acid develops a color with tertiary butyl peroxide for reading at 600 mμ.[8] The color body can be precipitated by dilution with water and dissolved in 70% sulfuric acid. The maximum is then shifted to 560 mμ. Beer's law is followed at peroxide concentrations less than 0.9 mg. per ml.

Procedure—To 1 ml. or less of sample containing not more than 18 mg. of *tert*-butyl-peroxide, add 10 ml. of 0.15% vanillin solution in 7:3 sulfuric acid solution. Incubate at 37° for 20 hours, dilute with 20 ml. of ethanol, and read at 600 mμ.

SESAMIN

Sesame oil mixed with acetic anhydride and a few drops of furfural on addition of concentrated sulfuric acid gives a brilliant red color which changes to blue-green.[9] The reaction is of sesamin. The acetic anhydride probably forms an acetyl derivative. Aldehydes other than furfural do not react.

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[8] Sven Arrhenius, *Acta Chem. Scand.* 9, 715-7 (1955).

[9] L. Pavolini and R. Isidoro, *Olii minerali, grassi e saponi, colori e vernici* 28, 137-40 (1951); Ascenzio Chindemi, *Boll. lab. chim. Provinciali (Bologna)* 9, 297-310 (1958).

Procedure—Dilute 0.5 gram of oil with benzene to 250 ml. To 5 ml. of this dilution add 5 ml. of acetic anhydride and 3-4 drops of 2% solution of furfural in 95% ethanol. Add drop by drop exactly 0.2 ml. of concentrated sulfuric acid. After 15 minutes dilute to 25 ml. with benzene and read at 620 mμ against a blank.

ROTENONE

Rotenone is read in the ultraviolet in ethanol solution.[10] The values at 284, 294, and 304 mμ are corrected by the Morton and Stubbs method.

Procedure—Extract a sample containing up to 50 mg. of rotenone with 100 ml. of acetone for 10 minutes with stirring. Filter or centrifuge and heat a 1-ml. aliquot at 60° to evaporate the solvent with a stream of air. Dissolve in ethanol, dilute to 100 ml. with ethanol, and read at 284 mμ, 294 mμ, and 304 mμ against ethanol. Calculate the absorbance of rotenone by correction for the absorbance of impurities such as by the method of Morton and Stubbs. Very tentatively A (corrected) $= 7.02A_{294} - 3.5A_{284} - 3.5A_{304}$.

FLAVONE, 3-PHENYLBENZO-4-PYRONE, AND DERIVATIVES

Flavones reduced by magnesium and hydrochloric acid produce an orange-yellow color; flavonols, red; and flavanones violet-red.[11] The compounds are separated from interferences by sorption on alumina gel. Since aluminum salts cause a slight change in color, standards must be run through the separation procedure. Beer's law is followed up to 1.2 mg. per 100 ml.

Procedure—Mix a 1-4 ml. sample containing 1-4 mg. of flavonic compound with 3 ml. of 15% aluminum chloride solution and 0.5 ml. of 1:1 ammonium hydroxide. Dilute to 10 ml. with water and centrifuge after 5 minutes. Decant the supernatant liquid and suspend the solid in 10 ml. of water containing a small amount of ammonium hydroxide. Centrifuge. Dissolve the solid in 2 ml. of concentrated hydrochloric acid. Add 3 ml. of 95% ethanol. Adjust the concentration of a sample to approximately 1-22 mg. in 5 ml. of 60% ethanol. Add 1 ml. of concentrated hydrochloric acid and 75 mg. of magnesium turnings. Read 10 minutes after the evolution of hydrogen stops. For this read the orange-red resulting from flavones against a luteolin standard. Likewise read the red from flavonols against a rutoside standard at 530 mμ or the violet-red from flavanones against hesperoside at 550 mμ.

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[10] Romeo Payfer, *J. Assoc. Offic. Agr. Chemists* 37, 630-3 (1954).

[11] R. Paris and J. Cornilleao, *Ann. pharm. franç.* 13, 192-9 (1955).

5-HYDROXY-2-(HYDROXY METHYL)-4-PYRONE, KOJIC ACID

Kojic acid reacts with ferrous ion to give a color at 500 mμ, stable for 6 hours[12] (Vol. III, pp. 179-80). Glucose, acetone and ethanol do not interfere. Oxalic, phosphoric, citric, sulfuric, tartaric, hydrochloric, nitric, lactic, gluconic, and succinic acids do interfere. The color is reduced by two-thirds in the presence of 0.5% of peptone.

Procedure—Adjust the pH of the sample to 6.2 and extract the kojic acid with ethyl acetate. Evaporate the ethyl acetate and take up the residue in water. Dilute to a known volume for use of an aliquot. Mix 10 ml. of sample solution containing 0.5-5 mg. of kojic acid with 1 ml. of 0.5% ferrous chloride solution and read at 500 mμ.

γ-BUTYROLACTONE

The reaction of γ-butyrolactone with ferric alum gives a color suitable for reading at 505 mμ.[13]

Procedure—As reagent mix 25 ml. of 20% hydroxylamine hydrochloride solution with 2 drops of an indicator containing 100 mg. each of phenolphthalein and thymolphthalein in 100 ml. of 95% ethanol solution. Add 12% sodium hydroxide solution until the reagent turns purple and then add 10-20 more drops of the indicator. Add a 5-ml. sample containing 0.7-1 mg. of γ-butyrolactone to 10 ml. of hydroxylamine hydrochloride-sodium hydroxide reagent. Heat for 20 minutes at 40° and cool in ice water. Shake with 10 ml. of a reagent containing 25 grams of ferric alum in 250 ml. of 1:8 nitric acid. After 10 minutes read at 505 mμ.

LIPID PEROXIDES

The methods of determination of lipid peroxides in the past have been titrametric. Now the method has been refined to a photometric measurement of the liberated iodine.[14] Also see the following method.

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[12] W. Hashida and T. Yamamoto, *Hakko Kagaku Zasshi* 30, 354-8 (1952).
[13] S. Mizukami and K. Nagata, *J. Pharm. Soc. Japan* 76, 1129-33 (1956).
[14] F. W. Heaton and N. Uri, *J. Sci. Food Agr.* 9, 781-6 (1958).

Procedure—The required apparatus is shown in Figure 8. A mixture of 2 parts of glacial acetic acid and one part of chloroform is deaerated in advance by bubbling nitrogen through it vigorously.

Add a sample expected to yield 0.02-5 micromoles of peroxide and 7.5 ml. of solvent. Add 0.5 ml. of 50% potassium iodide solution. The total volume must not exceed 10 ml. Mix and leave in the dark for 1 hour. Read at an appropriate wave length in the range of 370-500 mμ, the selection being made to correspond with the iodine content and calculate to 400 mμ for interpretation, Table 9.

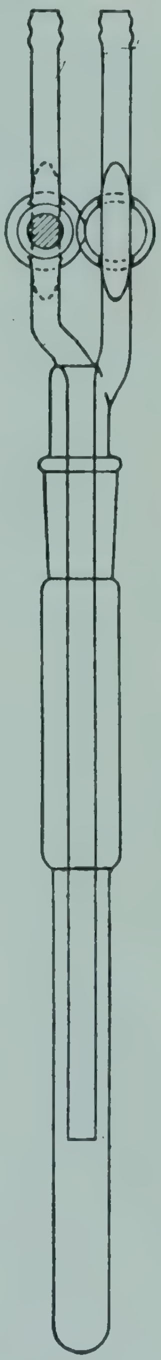

FIG. 8

Apparatus for microdetermination of lipid peroxides. One third size

ORGANIC PEROXIDES AND HYDROPEROXIDES

In a benzene-trichloracetic acid solution, benzoyl leuco methylene blue reacts with organic peroxides to produce the characteristic methylene blue color.[15] The peroxide oxidizes the reagent with zirconium naphthenate as the catalyst. Among the peroxides and hydroperoxides which react with the reagent are benzoyl peroxide, lauroyl peroxide, *p*-menthane hydroperoxide, and cumene hydroperoxide, all of which follow Beer's law up to 1 ppm., and *tert*-butyl hydroperoxide which deviates from Beer's law. Dialkyl peroxides such as di-*tert*-butyl peroxide do not react.

The reaction is heat-sensitive and temperatures over 30° cause erratic results. The color is stable in the dark for 24 hours but is sensitive to ultraviolet light and artificial light. Heat and light cannot be applied to increase the reaction rate since heat produces results which are not quantitative and light produces a decrease in reproducibility. The concentration of the zirconium naphthenate reagent is critical since an excess results in the reagent blank being excessive. As little as 0.5 mg. of active peroxide can be determined.

Among the organic peroxides which one may determine are the relatively unstable autooxidized lipides. Thus 3,4-dichloro-4,4′-dihydroxyphenylene-diamine reacts with peroxidized fats dissolved in an organic solvent in the presence of a ferric catalyst to produce dechlorophenolindophenol.[16] The color is read at 520 mμ. Results are probably high and reproducibility is poor. They have been found high in the presence of oxygen and low in its absence.[17] Addition of magnesium chloride is advantageous.[18]

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[15] M. I. Eiss and P. Giesecke, *Anal. Chem.* **31**, 1558-60 (1959); K. Ueberreiter and G. Sorge, *Angew. Chem.* **68**, 352-4 (1956).

TABLE 9. CONVERSION FACTORS FOR ABSORBANCE BY IODINE

mμ	*Conversion factor*	mμ	*Conversion factor*
380	2.26	450	0.204
390	1.56	460	0.145
400	1.00	470	0.103
410	0.670	480	0.070
420	0.476	490	0.048
430	0.355	500	0.034
440	0.273		

In the absence of oxygen attempts have been made to oxidize ferrous ion to ferric ion with organic peroxides and determine the ferric ion with thiocyanate (Vol. III, p. 176). However, results are much too high,[19] even though some workers have reported this method as the best available.

An intense blue is produced by organic peroxides with 4,4-diaminodiphenylamine sulfate in acetic acid.[20] The maximum absorption is at 640 mμ.

Procedure—*By benzyl leucomethylene blue.* Mix 15-20 ml. of 0.5% trichloroacetic acid solution in benzene with 0.3 ml. of 0.24% zirconium naphthenate solution in benzene, prepared by diluting 1 ml. of commercial 6% zirconium naphthenate solution to 25 ml. with benzene. Add 1 ml. of 0.05% benzoyl leucomethylene blue solution in benzene. To this mixture add the sample solution and dilute to 25 ml. with benzene. Mix and let stand in the dark at 24° for the time specified in the following tabulation.

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[16] S. Hartmann and J. Glavind, *Acta Chem. Scand.* **3,** 954-8 (1949); **9,** 497-508 (1955).

[17] C. H. Lea, *J. Sci. Food Agr.* **3,** 586-94 (1952).

[18] J. Glavind and S. Hartmann, *Acta Chem. Scand.* **9,** 497-508 (1955).

[19] B. Coleby, *Chem. & Ind.* (*London*) **1957,** 1123-4.

[20] K. Ueberreiter and G. Sorge, *Angew. Chem.* **68,** 352-4 (1956).

	Time for complete color development at 25°
tert-Butyl-hydroperoxide	30 minutes
Cumene hydroperoxide	40 minutes
p-Menthane hydroperoxide	2 hours
Lauroyl peroxide	5 hours
Benzoyl peroxide	30 hours

Read at 662 mμ against water. If the peroxide is unknown, check the completeness of reaction by measuring the absorbance until it no longer increases.

CHAPTER 7

TETROSES AND PENTOSES

BECAUSE many of the sugars react with the same reagents the group under pentoses in general includes important reactions for hexoses and higher carbohydrates. Some are referred to thereafter only by cross-reference. For determination of pentoses by iodine see page 183.

For determination of 2-*O*-methyl-*d*-xylose or 2,3,di-*O*-methyl-*d*-xylose by phenol in sulfuric acid see page 167. For determination of 2,3,4-tri-*O*-methyl-*d*-xylose by aminodiphenyl see page 168. For determination of lyxose in 79% sulfuric acid see page 168. For determination of lyxose by *N*-ethylcarbazole see page 168. For determination of ribulose by resorcinol and ferric ion see page 172.

For determination of fucose by anthrone in the presence of some other sugars see pages 179 and 185. For determination by thymol see page 197. For determination by periodate consumption see page 174. For determination by phenol in sulfuric acid see page 167. For determination in 79% sulfuric acid see page 168. For determination by carbazole see page 182, by *N*-ethylcarbazole page 168.

For determination of rhamnose by aminodiphenyl see page 168. For determination by anthrone see pages 175 and 195. For determination in 79% sulfuric acid see page 168. For determination by *N*-ethylcarbazole see page 168. For determination of adenosine by anthrone in the presence of some other sugars see page 195.

To determine desoxyadenosine, heat a solution of the order of 0.0125 mg. per ml. with 5% trichloroacetic acid for 15 minutes at 90° to hydrolyze to desoxyribose. Determine the latter by periodic acid and 2-thiobarbituric acid, page 177.

TETROSES

The reaction of pentoses and higher sugars with concentrated sulfuric acid gives furfural or a homolog. These give characteristic bands in the ultraviolet and combine with cysteine to give colored products. On standing at room temperature the wave length of maximum absorption changes. Tetroses and lower homologs do not so react and give no color when they are the only sugars in solution. Tetroses do combine with decomposition products of higher sugars to give yellow products.[1] This reaction is practically identical for *erythrose, threose,* and *erythrulose*. Higher saccharides influence the color developed with hexose as the second sugar. Glycolic aldehyde gives about 5% as much color as erythrose. Other homologs, trioses, and their phosphates do not interfere. Formaldehyde gives a noninterfering brown color. Erythrose-4-phosphate reacts like erythrose.

Procedure—*By fructose and sulfuric acid.* Mix 0.5 ml. of sample solution containing 0.1-0.4 mg. of tetrose per ml. with 0.5 ml. of 0.1% fructose solution. Add 4.5 ml. of 6:1 sulfuric acid, cooling in ice water. After taking to room temperature heat in boiling water for 3 minutes and cool. Let stand at room temperature overnight. Add 1.2 ml. of water and let stand for at least 8 hours. Read the yellow color at 438 mμ against a reagent blank.

By fructose, sulfuric acid, and cysteine. Proceed as before through ". . . 3 minutes and cool." Add 0.1 ml. of 3% solution of cysteine hydrochloride. Let stand at room temperature overnight and add 1.2 ml. of water. After about 10 hours read the greenish-yellow color at 460 mμ against a reagent blank which will show a faint purple color.

Carbohydrates in General

Phenol in sulfuric acid reacts to determine micro quantities of sugars, their methyl derivatives, oligosaccharides, and polysaccharides. By this method, pentoses, methyl pentoses, and uronic acids have an absorption maximum at 480 mμ, but certain methylated pentose sugars and their methyl glycosides show selective absorption at 415-420 mμ. Hexoses and their methylated derivatives have an absorption maximum at 485-490 mμ.[2] Specifically the maximum for each sugar and its derivatives is shown in Table 10.

The sugars must first be separated by paper chromatography with phenol-water, butanol-ethanol-water, ethyl acetate-acetic acid-water, methyl ethyl ketone-water, or other volatile solvent.

The intensity of the color is a function of the amount of phenol added up to a maximum. After the maximum color has been reached, the presence of additional phenol from the paper chromatographic separation or

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[1] Z. Dische and M. R. Dische, *Biochim. et Biophys. Acta* **27**, 184-8 (1958).

[2] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.* **28**, 350-6 (1956).

TABLE 10. WAVE LENGTH OF MAXIMUM ABSORPTION DEVELOPED WITH PHENOL AND SULFURIC ACID

Compound	*Wave length* mμ
d-Fructose	490
d-Glucose	485
Sucrose	490
5-Hydroxymethyl-2-furaldehyde	490
Starch	488
Dextran	488
d-Galacturonic acid	480
d-Mannurone	485
d-Glucurone	480
d-Galactose	487
d-Mannose	487
l-Arabinose	480
d-Xylose	480
l-Rhamnose	480
l-Fucose	480
Maltose	490
Raffinose	490
Lactose	490
2-*O*-Methyl-*d*-xylose	485
2,3-Di-*O*-Methyl-*d*-xylose	480
Methyl 2,3-di-*O*-methyl-*d*-xyloside	415, 480
2,3,5-Tri-*O*-methyl-*l*-arabinose	415
Methyl 2,3,5-tri-*O*-methyl-*l*-arabinoside	415
2,3-Di-*O*-methyl-*d*-glucose	485
2,3,6-Tri-*O*-methyl-*d*-glucose	485
2,3,4,6-Tetra-*O*-methyl-*d*-glucose	485
2,3-Di-*O*-methyl-*d*-mannose	485
2,3,6-Tri-*O*-methyl-*d*-mannose	485
2,3,4,6-Tetra-*O*-methyl-*d*-galactose	485

other sources has little effect as long as the amount of phenol added to sample and standards is controlled. Therefore, the amount of color produced at a constant phenol concentration is proportional to the amount of sugar present.

Aldopentoses, aldohexoses, methyl aldopentoses, hexuronic acids, and certain oligosaccharides are estimated in the concentration range of 0.02-0.5 mg. per ml. with an acetic acid solution of aminodiphenyl.[3] The reagent condenses with the sugar to form a characteristic color for each class of sugars as follows: aldohexoses and some methylated derivatives, green; aldopentoses, yellowish brown; hexuronic acids, yellow; rhamnose and some methylated aldohexoses, light brown; di- and tri-saccharides, green, except maltose which is green-yellow. The reagent is stable for 2-3 days when kept in the dark. Potassium sulfite may be added as a stabilizing agent but the potassium sulfite causes all colors formed to fade to light brown within a few hours, without affecting their absorbances at the wave lengths shown in Table 11. The colors are otherwise stable for 24 hours. Galactose, glucose, mannose, arabinose, ribose, and xylose show the same absorptivity.

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[3] T. E. Timell, C. P. J. Glaudemans, and A. L. Currie, *Ibid.* **28**, 1916-20 (1956).

TABLE 11. OPTICAL PROPERTIES OF GLYCOSYLAMINES OF VARIOUS SUGARS

Sugar	*Color*	*Wave length for maximum absorption,* mμ	*Minutes of heating necessary for maximum absorption*
Arabinose	Brownish-yellow	370	30
Ribose	Brownish-yellow	370	30
Xylose	Brownish-yellow	375	30
Galactose	Green	380	45
Glucose	Green	380	45
Xylose	Green	380	45
Rhamnose	Brown	375	45
Galacturonic acid	Yellow	380	30
Glucuronolactone	Yellow	375	30
Cellobiose	Green	380	60
Lactose	Green	375	90
Maltose	Greenish-yellow	380	120
Melibiose	Greenish-yellow	375	120
Sucrose	Greenish-yellow	380	60
Melezitose	Green	380	120
Raffinose	Green	380	120
2-*O*-Methyl-*d*-glucose	Brownish-yellow	370	90
3-*O*-Methyl-*d*-glucose	Green	380	45
6-*O*-Methyl-*d*-glucose	Green	375	60
2,3,4,6-Tetra-*O*-methyl-*d*-glucose	Green	375	120
2,4,6-Tri-*O*-methyl-*d*-galactose	Yellow	370	120
2,3,4-Tri-*O*-methyl-*d*-xylose	Brown	375	120

A wave length of 380 mμ can be used for all the sugars but as shown in Table 11 the time of heating necessary to produce maximum absorbance varies from 30 minutes for aldopentoses and hexuronic acids to 120 minutes for oligosaccharides and higher methylated sugars.

Cellobiose and lactose are estimated with the same accuracy as the monosaccharides, but maltose, melibiose, and sucrose require larger quantities for similar absorbance. Melezitose and raffinose exhibit lower absorbancies. Fructose cannot be determined by this method. Beer's law is followed.

As little as 0.001 mg. of total carbohydrate per ml. of sea water is determined by *N*-ethylcarbazole. Pentoses, hexoses, and their polymers yield the same amount of color per unit weight.[4] Exceptions are sorbose, xylose, and fructose which give about double the color of arabinose employed as a standard. Fucose and rhamnose yield substantially less color than the standard. Glucosamine hydrochloride, ascorbic acid, and malic acid do not give color below 10 mg. per liter.

The pentoses, arabinose, xylose, ribose, and lyxose, show a peak at 544 mμ with a secondary peak at about 600 mμ. The hexoses, glucose, fructose, galactose, mannose, and sorbose show a single maximum at 600 mμ. Their di- and oligosaccharides, maltose, lactose, sucrose, turanose, trehalose, melibiose, melezitose, and α-methyl glucoside also show a single maximum at 560 mμ. The methylated sugars, fucose and rhamnose, and ascorbic acid show a single maximum at 548-550 mμ. The best general value for reading is 560 mμ.

The reagent solution decomposes upon exposure to open sunlight and ultraviolet light, and should not be used after 48 hours. Since the reagent turns green in the presence of a high nitrate concentration, all glassware used must be soaked overnight in sulfuric acid and rinsed in triple-distilled water. Samples remain constant in the dark for 4 hours and increase in color in the light.

The blue color of carbohydrates in 79% sulfuric acid is suitable.[5] The reaction is applicable to *d*-xylose, *d*-arabinose, *d*-ribose, *d*-lyxose, *l*-fucose, *l*-rhamnose, *d*-glucose, *d*-mannose, *d*-galactose, *d*-fructose, *l*-sorbose, *d*-glucurone, *d*-galacturonic acid, *N*-acetyl-*d*-glucosamine, *d*-glucosamine hydrochloride, *d*-mannitol, *meso*-inositol, adonitol, sucrose, maltose, trehalose, lactose, melibiose, raffinose, melezitose, starch, glycogen, and inulin. The color of different members of the series is additive although the peaks are not identical. The products of reaction with pentoses, hexoses, and polyoses are not the same.[6] Products formed from pentoses are furfural, formaldehyde, crotonic aldehyde, and acetaldehyde.

Aldopentoses, aldohexoses, hexuronic acids, and 6-desoxyaldohexoses separated by paper chromatography are colored by spraying with *p*-anisidine hydrochloride,[7] eluted with methanol, and the absorbance measured. The possible general reaction involved is an initial degradation to furfural followed by a condensation with *p*-anisidine to form the characteristic red-colored compound.[8] Aldopentoses and hexuronic acids exhibit absorption maxima at 510 mμ. Aldohexoses and 6-desoxyaldohexoses form brown compounds with maxima at 400 and 385 mμ respectively. The colors formed are relatively stable with the exception of the color formed between the reagent and aldopentoses or hexuronic acids. Although stannous chloride is added to increase color stability, the measurements should be made within 30 minutes of color development. Beer's law is followed over a range of 0.005-0.05 mg. as extracted into 3 ml. of methanol reagent.

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[4] Z. P. Zein-Eldin and B. Z. May, *Ibid.* **30,** 1935-41 (1958).

[5] M. Ikawa and C. Niemann, *J. Biol. Chem.* **180,** 923-31 (1949); *Arch. Biochem. and Biophys.* **31,** 62-71 (1951).

[6] R. M. Love, *Biochem. J.* **55,** 126-32 (1953); F. A. H. Rice and L. Fishbein, *J. Am. Chem. Soc.* **78,** 1005-9, 3731-4 (1956); F. A. H. Rice, *Ibid.* **78,** 6167-8 (1956).

Individual and total pentoses are determined in sugar mixtures by thionalide reagent in concentrated sulfuric acid. When one pentose is present it is identified by the ratio of the absorbance at 540 mμ divided by that at 580 mμ, since this ratio is specific for each sugar.[9]

Benzidine is a suitable reagent for the determination of 0.1-0.6 mg. per ml. of aldopentoses, methyl aldopentoses, aldohexoses, hexuronic acids, methylated sugars, and disaccharides.[10] The reagent is unstable and requires the addition of a reducing agent such as stannous chloride or potassium sulfite. Unless specially treated, papers give high blanks. Below 0.1 mg. per ml. it is nonlinear. Color is also given by hexose diphosphate, 2-desoxy-*d*-galactose, dihydroxyacetone, acetone, formaldehyde, α-oxoglutaric acid, and other compounds with a free or easily accessible carbonyl group. *d*-Fructose gives a color only at high concentrations. There is no significant color with glycine, tryptophan, tyrosine, asparagine, cystine, hydroxyproline, urea, hexitols, or calcium gluconate. At 0.5 mg. per ml. *d*-glucosamine gives a faint color.

Anthrone determines many sugars including xylose, arabinose, ribose, glucose, fructose, and galactose. After hydrolysis of the sample it also determines xylan, sucrose, maltose, cellobiose, glycogen, inulin, starch, pectin, and cellulose.[11] Methods for many of these are given under the specific compounds. Classes are differentiatable by their time of reaction. Pentoses reach their maximum color rapidly and fade quickly. Ketoses reach their maximum color quickly but fade less rapidly than pentoses. Aldoses reach the maximum quickly and fade even more slowly.

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[7] L. Hough, J. K. N. Jones, and W. H. Wadman, *J. Chem. Soc.* **1950,** 1702-6.

[8] John B. Pridham, *Anal. Chem.* **28,** 1967-8 (1956).

[9] H. Masamune and K. Ogawa, *Tohoku J. Exptl. Med.* **60,** 23-31 (1954); H. Masamune and M. Sakamoto, *Ibid.* **63,** 357-62 (1956).

[10] L. Hough, J. K. N. Jones, and W. H. Wadman, *J. Chem. Soc.* **1950,** 1702-6; J. K. N. Jones and J. B. Pridham, *Nature* **172,** 161 (1953) (1 page); *Biochem. J.* **58,** 288-90 (1954).

[11] Mrs. László Márkus, *Agrokémia és Talajtan* **3,** 227-34 (1954).

Thus with 0.1% anthrone in sulfuric acid reacted with an equal volume of the sugar solution the maximum color at 560-620 mμ is reached by glucose after 9 minutes and remains constant thereafter for 11 minutes, and decreases gradually thereafter.[12] Arabinose reaches its maximum in 2 minutes, decreases to 26% of its maximum after 5 minutes, and has lost the blue color in 10 minutes. This color intensity curve does not parallel furfural formation. Therefore the fading is due to instability of the blue pigment formed rather than to instability of furfural formed. In support of this furfural itself with the reagent reaches a blue having a maximum at 600 mμ promptly and fades promptly. Table 12 gives a series of color intensities as developed at constant time. The importance of accurate standardization of conditions of heating, time of heating, and conditions of cooling are emphasized by these factors. Rate of addition of the reagent can also affect the intensity obtained.[13]

Table 12. Relative Color Intensity by 1 ml. of 0.1% Anthrone Reagent in 72% Sulfuric Acid with 1 ml. of Sugar Solution Developed for 10 Minutes. Values Designated as Hydrolyzed Are after Hydrolysis with 1:17 Sulfuric Acid

Ribose	8
Arabinose	6
Xylose	6
Rhamnose	95
Glucose	10
Fructose	99
Mannose	60
Galactose	55
Sucrose	111
Lactose	72
Maltose	98
Glycogen	110, hydrolyzed 106
Dextran	105, hydrolyzed 95
Dextrin	97
Amylose	107, hydrolyzed 104
Amylopectin	— hydrolyzed 100
Soluble starch	96, hydrolyzed 94

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[12] Kenjiro Yamamoto, *Nippon Yakurigaku Zasshi* 52, 602-14 (1956).

[13] Tomoya Nakata, *Tokyo Jikeikai Ika Daigaku Zasshi* 72, 464-70 (1957).

Ketopentoses, ketohexoses, trioses, and glycolic aldehyde react with carbazole in strong sulfuric acid.[14]

When simple pentoses and hexoses are to be separated by paper chromatography, desirable solvents are ethyl acetate-acetic acid-water or ethyl acetate-pyridine-water.[15]

The various methods which involve conversion to furfural have weaknesses when applied to wheat bran and peanut hulls.[16] The extracted color has poor stability. The orcinol method is lengthy.

As a general reaction periodate will oxidize any compound having a terminal-CH_2OH group to give formaldehyde.[17] The latter can then be determined as an indirect measure of the compound.

Samples—*Separation by paper chromatography.* An 8 × 22 inch sheet of filter paper is used, with a similar one as the blank. Draw two lines lengthwise 1.5 inches from the edge of the paper. Draw lines across 1 inch from the top and 3.5 inches from the bottom.

Apply a solution containing about 3-4 mg. of sugar to the paper along the 3.5-inch line. At the same time apply qualitatively amounts of the same solution to the paper on the 1.5-inch marker strips along the sides. Develop the chromatogram for 24 hours with phenol saturated with water as the solvent. Dry the paper for 24 hours. Cut off the marginal marker strips and spray with *p*-anisidine trichloroacetic acid solution to locate the sugars on the chromatogram. Reassemble with the center strip and mark the lines of demarkation. Cut out the appropriate sections containing the sugars and corresponding sections from the blanks. Extract each sugar area and its blank with water and filter. Develop the solutions so obtained by the phenol-sulfuric acid method.

Procedure—*By phenol-sulfuric acid.*[18] Add 0.05 ml. of 80% phenol solution to a 2-ml. aliquot of sample containing 0.01-0.07 mg. of the sugar. Alternatively use 1 or 2 ml. of sample with 1 ml. of 5% aqueous phenol. Add 5 ml. of concentrated sulfuric acid rapidly, directing at the liquid surface to obtain good mixing. Let stand for 10 minutes and incubate at 25°-30° for 10-20 minutes. Read at 490 mμ for hexoses and at 480 mμ for pentoses and uronic acids against a reagent blank.

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[14] Z. Dische and E. Borenfreund, *J. Biol. Chem.* **192,** 583-7 (1951).

[15] M. A. Jarmyn and F. A. Isherwood, *Biochem. J.* **44,** 402-7 (1949).

[16] Yoshiyuki Matsuo, *J. Fermentation Technol.* (*Japan*) **33,** 509-13 (1955).

[17] S. L. Thompsett and D. C. Smith, *Analyst* **78,** 209-16 (1953).

[18] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.* **28,** 350-6 (1956).

By o-*aminodiphenyl.*[19] To a 1-ml. aliquot of sample solution containing 0.01-0.5 mg. of sugar add 5 ml. of 0.4% *o*-aminodiphenyl solution in glacial acetic acid. Heat at 100° for the time required in Table 11. Cool in water and read at the appropriate wave length against a reagent blank similarly treated.

By N-*ethylcarbazole. Sea water.* The reagent may require purification. If so heat 1 liter of 95% ethanol and slowly add 250 grams of *N*-ethylcarbazole in small portions so that they dissolve before they have time to melt, as further decomposition may result. After crystallization discard the first mother liquor. Redissolve the crystals in hot ethanol as before and again allow to crystallize. Treat the mother liquor with an equal volume of water to precipitate the remaining *N*-ethylcarbazole. Combine the crystals and the precipitate. Repeat the procedure of crystallization from hot ethanol and precipitation with water until pure white needle-shaped crystals result. Remove sorbed alcohol by drying at 55°.

Add 5 ml. of 0.1% *N*-ethylcarbazole solution in concentrated sulfuric acid to a 3-ml. sample containing 1-10 mg. of total carbohydrate per liter. One minute later, add 22 ml. of additional reagent. Heat at 70° for 30 minutes and bring to room temperature in a refrigerator. Read against water at 560 mμ or 525 mμ, using a 5-cm. cell for samples containing less than 4 mg. of carbohydrate per ml. The color is stable in the dark but increases with time in the light.

By 79% sulfuric acid. Mix 9 ml. of 85% sulfuric acid with 1 ml. of aqueous sample at 0°. Keep the resulting solution in 79% sulfuric acid for 2 hours at 25° or 15 minutes at 100° and read.

By p-*anisidine hydrochloride.*[20] As a reagent, dissolve 1 gram of crystalline *p*-anisidine hydrochloride in 10 ml. of absolute methanol and dilute to 100 ml. with 1-butanol. Add 0.1 gram of sodium hydrosulfite. Store in a refrigerator.

To chromatographic paper, apply 3-4 spots of standard mixed sugar solutions containing the same sugars as in the sample in the range of 0.005-0.05 mg., and apply similar spots with the unknown solutions. Develop with a 1-butanol-pyridine-water solvent in a 6:4:3 ratio or an ethyl acetate-acetic acid-water solvent in a 9:2:2 ratio for a minimum of 20 hours. Air dry and spray uniformly with the *p*-anisidine hydrochloride reagent. Heat at 130° for 10 minutes in an oven.

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[19] T. E. Timell, C. P. J. Glaudemans, and A. L. Currie, *Ibid.* 28, 1916-20 (1956).

[20] Jotin B. Pridham, *Ibid.* 28, 1967-8 (1956).

Cut out as small areas as possible of the sugar spots together with suitable blanks, keeping the area of the paper constant for each sugar series. Elute from the paper with 3 ml. of methanol reagent prepared by adding 90 ml. of methanol to 1 gram of stannous chloride as a stabilizing agent dissolved in 5 ml. of water. Read within 30 minutes as follows: aldopentoses and hexuronic acids, 510 mμ; aldohexoses, 400 mμ; and 6-desoxyaldohexoses 385 mμ.

By thionalide. Pentoses.[21] To a 0.5-ml. sample containing 0.01-0.08 mg. of pentose which has been cooled to 0°, add 3 ml. of concentrated sulfuric acid and heat for 5 minutes at 100°. Cool in ice and add 3 ml. of concentrated sulfuric acid containing 1 mg. of thionalide. Maintain at 20° for 48 hours and read at 540 and 580 mμ.

Methyl pentoses. To a 0.5-ml. sample containing 0.1-0.4 mg. of methyl pentose add 3.3 ml. of 1:1 sulfuric acid containing 1 mg. of thionalide. Heat at 100° for 5 minutes, cool in ice, and keep at 20° for 4.5 hours. Read at 487 and 520 mμ and determine the methyl pentose present by the ratio of absorption at 425 mμ to that at 520 mμ.

By benzidine.[22] To a 1-ml. sample solution containing 0.02-0.6 mg. of sugar, add 5 ml. of reagent, prepared by dissolving 0.2 gram of benzidine in 100 ml. of glacial acetic acid containing 0.1% of stannous chloride. Heat at 100°, cool rapidly, and read at 425 mμ.

By resorcinol.[23] To a sample containing 0.01-0.1 mg. of ketose, add 3 ml. of 0.05% resorcinol solution in absolute ethanol and 3 ml. of concentrated hydrochloric acid containing 0.216 gram of ferric ammonium sulfate per liter. Heat at 77-83° for 40 minutes, or 60 minutes if sorbose is present. Cool rapidly in ice and read after 5 minutes at 640 mμ for ketopentoses and 480 mμ for ketohexoses.

ALDOSACCHARIDES

The color reaction of *o*-toluidine in strong acetic acid is applicable to aldosaccharides in blood and urine.[24] The amount of water present in the final solution must not exceed 10%.

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[21] H. Masamune and M. Sakamoto, *Tohoku J. Exptl. Med.* **63,** 357-62 (1956).

[22] J. K. N. Jones and J. B. Pridham, *Biochem. J.* **58,** 288-90 (1959).

[23] R. G. Kulka, *Biochem. J.* **63,** 542-8 (1956).

[24] Eric Hultman, *Nature* **183,** 108-9 (1959).

Procedure—*Blood.* Mix 0.1 ml. of whole blood with 1 ml. of 3% trichloroacetic acid solution and centrifuge. Mix 0.2 ml. of the clear centrifugate with 3 ml. of 6% *o*-toluidine in glacial acetic acid. Heat at 100° for 8 minutes and cool. Read against a reagent blank at 625 mμ.

Urine. Mix 0.004 ml. of urine with 3 ml. of 2% solution of *o*-toluidine in glacial acetic acid. Boil for 8 minutes, cool, and read at 625 mμ against a reagent blank.

XYLOSE

Xylose, ribose, and arabinose are determined simultaneously with glucose or galactose by heating these sugars with orcinol in the presence of sulfuric acid or hydrochloric acid. Adding ferric chloride causes the pentoses to give an additional strong absorption band in the red at 660 mμ.[25] Anhydrous orcinol is required since any water present produces a high blank. The orcinol used as reagent should be freshly recrystallized from hot water containing charcoal. The concentration of both pentoses and hexoses is read from a nomogram, Figure 9. For separation of aldo- and ketohexoses, pentoses, and uronic acids in microgram quantities by paper chromatography a 3:1:3 mixture of ethyl acetate-acetic acid-water is recommended.[26]

For determination of xylose by phenol and sulfuric acid see page 167. For determination by aminodiphenyl see page 168. For determination by anthrone see page 175.

For determination of xylose in 79% sulfuric acid see page 168. For determination by *N*-ethylcarbazole see page 168. For determination in 90% sulfuric acid see page 179.

When glucose, galactose, lactose, xylose, and arabinose are separated on a paper chromatogram they are developed on the paper with 9.3% diphenyl amine—9.6% citric acid in 50% ethanol. Thereafter the eluate of the spots is read at 420 mμ.[27]

Procedure—*By orcinol.* To a 1-ml. portion of sample containing 0.005-0.025 mg. of sugar, add 10 ml. of 0.2% orcinol solution in 2:1 sulfuric acid. Mix, cover, and heat for 15 minutes at 100°. Cool rapidly to room temperature and read after 30 minutes at 425 mμ.

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[25] W. R. Fernell and H. K. King, *Analyst* 78, 80-3 (1953).

[26] I. H. Bath, *Ibid.* 83, 451-5 (1958).

[27] J. W. Date, *Scand. J. Clin. and Lab. Invest.* 10, 149-54 (1958).

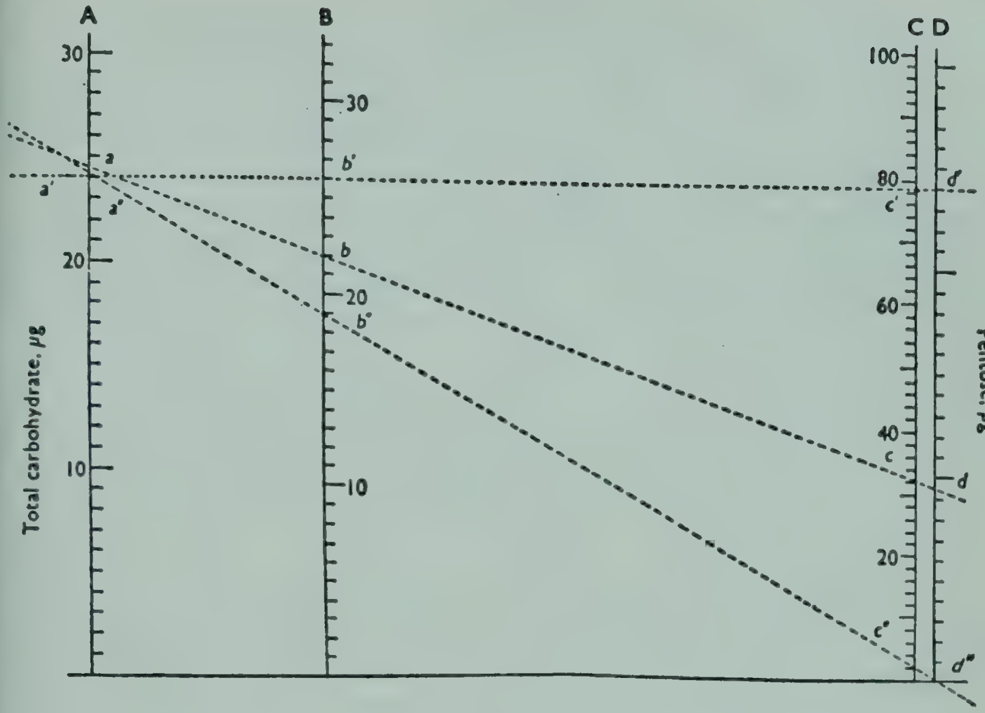

Fig. 9

Nomogram for calculating quantities of pentose and hexose from colorimeter readings

A, total carbohydrate (pentose plus hexose), μg per ml; B, colorimeter readings, orcinol-sulphuric acid method; C, colorimeter readings, orcinol-ferric chloride method; D, pentose, μg per ml

By orcinol-ferric chloride. As a reagent dissolve 0.15 gram of ferric chloride and 0.4 gram of orcinol in 10 ml. of water and dilute to 200 ml. with 3:7 hydrochloric acid. To a 2-ml. sample containing 0.005-0.025 mg. of sugar, add 6 ml. of reagent. Mix, close the tube, and heat at 100° for 20 minutes. Cool rapidly to room temperature. Read after 30 minutes at 660 mμ. Calculate the amount of pentose or hexose present from a nomogram, Figure 9.

Xylulose

Xylulose, a ketopentose, is differentiated from ketohexoses by resorcinol. The ketopentoses give a green color with resorcinol and ferric ion having a maximum absorption around 640 mμ.[28] Under the same conditions ketohexoses give a red color absorbing in the 415-480 mμ region. The color developed by heating at 80° is 95% of the maximum at 640 mμ after 40 minutes. Variation in quality of the absolute ethanol affects the results. Beer's law is not followed at 640 mμ. Ribulose gives a result similar to that of xylulose. Monoacetone xylulose gives a color identical with that of xylulose at molar equivalents.

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[28] J. S. D. Bacon and D. J. Bell, *Biochem. J.* 42, 397-405 (1948); M. J. McCorkindale and N. L. Edson, *Ibid.* 57, 518-23 (1954); R. G. Kulka, *Ibid.* 63, 542-8 (1956).

Fructose gives only a minor amount of color at 640 mμ. At 480 mμ its color is intense. Therefore at that wave length the reaction is also applied to determination of fructose. Inulin gives 93% of the color of fructose at that wave length. At a 1:1 concentration, glucose causes only a 1% error in the fructose determination. Sorbose gives 73% of the color of fructose and tagalose 83% of that of fructose at 480 mμ. Sorbose is preferably heated for 60 minutes. Perseulose and mannoheptulose have maxima at 430 mμ and 615 mμ. Generally these ketohexoses obey Beer's law up to 0.01 mg. per ml.

Ketoheptoses interfere with both the determination of ketohexoses and ketopentoses. Dihydroxyacetone gives a pink with a maximum at 520 mμ causing fructose and xylulose values to be low. Glucose, galactose, and lactose produce 1% of the color of fructose and do not interfere with determination of ketopentoses. The interference with aldopentoses at 640 mμ is 0.8% by arabinose, 2.3% by xylose, and 3.4% by ribose. Sorbitol, mannitol, and gluconic acid give no appreciable color.

The color of the fructose product is not altered in 5 hours. That of xylulose decreases 3% in 0.5 hour. Empirical correction is necessary if the amount of fructose is more than twice that of xylulose. The method is satisfactorily applicable to blood but interfering substances in urine are too great.

Procedure—*Blood.* Lake 1 ml. with 7 ml. of water. Add 1 ml. of fresh 7% metaphosphoric acid solution and mix. Filter the precipitated proteins To 2 ml. of sample solution containing 0.01-0.1 mg. of xylulose, add 3 ml. of 0.05% solution of resorcinol in absolute ethanol followed by 3 ml. of 0.0216% solution of ferric ammonium alum. Mix and heat at 80° ± 3° for 40 minutes. Cool in ice water and read at 640 mμ about 5 minutes after cooling. For ketohexoses read at 480 mμ.

RIBOSE

An indirect estimation of the concentration of ribose (Cf. Vol. III, pp. 190-1) is made by reading the consumption of periodate in the ultraviolet during an arbitrary time interval. Since most monosaccharides possess distinctive rates of periodate oxidation, not only *d*-ribose but also *d*-glucose, *d*-galactose, *d*-mannose, 2-desoxyribose, *l*-fucose, *l*-sorbose, *d*-sorbitol, *d*-fructose, and *d*-glucosamine are determined after separation by chromatography.[29] The technic is also applicable to four disaccharides; maltose, cellobiose, lactose, and sucrose.

Solvents used in the paper chromatographic separation of the sugars may interfere and are eliminated by steaming the chromatogram in an autoclave for 10 minutes at 90-100° before eluting the sugars. Two possible sources of error from the paper must be corrected for. The first is extractable substances which absorb at 227 mμ. This is eliminated by a reading on the sample and periodate at zero time. The second is oxidizable substances present in the extract. To correct this elute and assay blank sections of paper in the same manner as the standards and unknown. This correction, expressed as the difference in absorbance between the paper blank at zero hour and assay hour is added to the readings.

The anthrone reagent commonly used to determine hexoses is applied to the determination of ribose and other pentoses provided the reaction mixture is cooled in an ice bath immediately upon addition of the anthrone to keep the blue-green color stable.[30] Solutions not cooled in this way change rapidly to an amber color with no distinct maximum.

Ribose and ribonucleic acid exhibit a maximum blue color at 620-625 mμ while desoxyribose and desoxyribonucleic acid exhibit a red color with maxima at 565 and 530 mμ with anthrone.[31] The color of the nucleic acid is due to the reaction of the pentoses contained. That from 0.04-0.18 mg. of ribose is stable for 15 minutes after reaching room temperature and that from 0.2-0.7 mg. of ribonucleic acid is stable for 30 minutes. Additional heating over 2.5 minutes at 100° causes the ribose and ribonucleic acid colors to fade. However, a longer heating time of 6-10 minutes is required for the maximum color development of desoxyribose and desoxyribonucleic acid. Therefore 10 minutes is chosen not only for maximum color development but also to allow the absorbances of ribose and ribonucleic acid to decrease to a minimum. Desoxyribose and desoxyribonucleic acid are stable for several hours. Beer's law is followed from 0.025-0.1 mg. of desoxyribose, up to 0.3 mg. of ribose, up to 0.4 mg. of desoxyribonucleic acid, and up to 0.5 mg. of ribonucleic acid.

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[29] Laurence H. Frommhagen, *Anal. Chem.* 28, 1202-4 (1956).

[30] R. R. Bridges, *Anal. Chem.* 24, 2004-5 (1952); Tomoya Nakata, *Tokyo Jikeikai Ika Daigaku Zasshi* 72, 464-70 (1957).

[31] N. D. Gary and R. E. Klausmeier, *Anal. Chem.* 26, 1958-60 (1954).

Ribose and desoxyribose do not interfere with each other in their individual determinations, since the absorbance values for mixtures at 565 and 620 mμ are additive. The pentoses and nucleic acids are also determined in the presence of each other. Hexoses interfere since they give similar reactions of greater sensitivity with anthrone. Ribo- or desoxyribonucleic acid are determined in the presence of each other.

Ribose is determined in the presence of other sugars by phloroglucinol Phosphoric esters of ribose, adenosine monophosphate, yeast, adenylic acid, coenzymes I and II, and ribose-5-phosphate yield the same red color and absorption spectrum as ribose.[32]

For determination of ribose by thymol see page 197. For determination by anthrone in the presence of some other sugars see page 195. For determination by orcinol see pages 170 and 210. For determination by aminodiphenyl see page 168. For reading ribose and glucose in the same solution see pages 188 and 199. For determination in 90% sulfuric acid see page 179. For determination in 79% sulfuric acid see page 168. For determination by *N*-ethylcarbazole see page 168.

Samples—*Separation of* d-*ribose,* d-*glucose,* d-*galactose,* d-*mannose,* 2-*desoxyribose,* l-*fucose,* l-*sorbose,* d-*sorbitol,* d-*fructose, and* d-*glucosamine.* Spot the chromatographic paper and run two-dimensionally in collidine, a substituted pyridine, saturated with water and in a 4:1:5 mixture of water-saturated butanol, acetic acid, and water. Steam in an autoclave for 10 minutes at 90-100° to remove solvents. Cut out the areas containing the sugars, fold, and immerse in 10 ml. of water for 4 hours with continued shaking at 37°. Centrifuge the eluate to remove paper lint and develop a 1-ml. aliquot by periodate consumption.

Procedure—*By periodate consumption.*[33] Pipet 1 ml. of sample solution containing up to 0.02 mg. of the sugar to be determined into 5 ml. of 0.214% anhydrous sodium periodate solution. This periodate solution must be allowed to age 24 hours before use. Read the absorption at 227 mμ at once against a reagent blank in order to correct for background absorption. Read again at 3 hours. Correct the reading for the background absorption at zero time and for a blank run on an extract of the paper after 3 hours, before reading from the curve for the corresponding sugar. In some cases the reading at a longer period of time, such as 25 hours, is advantageous.

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[32] Z. Dische and E. Borenfreund, *Biochim. et Biophys. Acta* 23, 639-42 (1957).

[33] Laurence H. Frommhagen, *Anal. Chem.* 28, 1202-4 (1956).

By anthrone. d-*Ribose,* l-*arabinose, or* d-*xylose.* Take a 2-ml. sample containing 0.01-0.1 mg. of sugar per ml. in a 16 × 144 mm. test tube. Add carefully with a pipet as a lower layer 4 ml. of 0.05% solution of anthrone in concentrated sulfuric acid. Quickly mix by oscillating. The temperature will rise to about 95°. Immerse in an ice bath for 5 minutes. Let come to room temperature and read at 620 mμ.

Ribose, desoxyribose, ribonucleic acid, and desoxyribonucleic acid.[34] Adjust an aliquot of sample containing 0.025-0.1 mg. of desoxyribose, up to 0.3 mg. of ribose, up to 0.4 mg. of desoxyribonucleic acid, and up to 0.5 mg. of ribonucleic acid to 2 ml. with water and cool in an ice bath. Holding the tubes in an inclined position slowly add 4 ml. of cold 0.05% anthrone solution in concentrated sulfuric acid so that a layer forms below the sample. Return to the ice bath and thoroughly mix. Cover and heat at 100° for 2.5 minutes for ribose or ribonucleic acid and 10 minutes for desoxyribose or desoxyribonucleic acid. Cool to room temperature using an ice bath and read at 620-625 mμ for ribose or ribonucleic acid and at 656 and 530 mμ for desoxyribose or desoxyribonucleic acid, against a reagent blank.

To calculate the concentration of desoxyribonucleic acid or ribonucleic acid in mixtures of both, assume the absorbance at 565 mμ for the 10 minutes reaction is due to desoxyribonucleic acid alone. Therefore

$$\mathrm{DNA} = \mathrm{D}^{565}. \qquad (1)$$

The absorbance of the 2.5-minute reaction at 620 mμ is due to the sum of both nucleic acids. By subtracting the absorbance due to desoxyribonucleic acid, ribonucleic acid is then determined as follows

$$\mathrm{RNA} = (\mathrm{R} + \mathrm{D})^{620} - \mathrm{D}^{620}. \qquad (2)$$

To determine the amount of desoxyribonucleic acid color present in the mixture at 620 mμ, multiply the value measured at 565 mμ by a conversion factor K

$$\text{or} \quad \mathrm{D}^{620} = \mathrm{KD}^{565} \qquad (3)$$

where $K =$ the ratio of the absorbance values obtained with standard solutions of DNA at 620 and 565 mμ after reactions of 2.5 and 10 minutes respectively or

$$K = \frac{\mathrm{DNA}^{620}}{\mathrm{DNA}^{565}}.$$

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[34] N. D. Gary and R. E. Klausmeier, *Ibid.* 26, 1958-60 (1954).

Substitution of the values for K and D^{620} in the equations numbered (2) and (3) above gives ribonucleic acid.

By phloroglucinol.[35] Treat a 0.4-ml. aliquot of sample containing 0.004-0.04 mg. of ribose with 5 ml. of a mixture of 110 ml. of glacial acetic acid, 2 ml. of concentrated hydrochloric acid, 1 ml. of 0.8% glucose solution, and 5 ml. of 5% phloroglucinol solution at 100°. Read the red color formed at 552 mμ.

DESOXYRIBOSE

Desoxyribose yields a strongly fluorescent product when reacted with 3,5-diaminobenzoic acid in concentrated mineral acids. It is not necessary to separate ribose nucleic acid since the reaction is specific for desoxyribose.[36] The purine desoxynucleotides in the central nervous system tissues are hydrolyzed by the strongly acid 3,5-diaminobenzoic acid and the desoxyribose is liberated to react with the reagent.

Lipides which fluoresce with the reagent are extracted with alcoholic potassium acetate. Possible contamination by acid-soluble α-methylene aldehydes is eliminated by precipitation with trichloroacetic acid. Although acetaldehyde, undecylic acid, and undecylenic aldehyde cause fluorescence with the reagent, they do not interfere under the conditions of the method. In concentrations of 7-2000 millimicromoles of ribose, glucose, glucosamine, inositol, adenine, uracil, xanthine, thymine, cytidene, cytosine, cytidylic acid, uridine, uridylic acid, adenosine, adenylic acid, guanine, or guanylic acid there is no fluorescence with the acid reagent. Perchloric acid is added since the method has maximum sensitivity in the presence of this acid. As little at 0.0000024 mg. of desoxyribosenucleic acid in 50 microliters may be determined.

Desoxyribose (Cf. Vol. III, p. 191) or desoxyribonucleic acid in the range of 25-250 mg. per liter are determined by the blue color formed with diphenylamine.[37] Oxidation of 2-desoxy sugars with periodate gives malonic aldehyde.[38] This reacts with 2-thiobarbituric acid [39] for estimaion.[40] The color is stable for at least one hour. Beer's law holds up to at east 0.0025 mg. per ml. The reaction is complete.

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[35] Z. Dische and E. Borenfreund, *Biochim. et Biophys. Acta* **23**, 639-42 (1957).

[36] J. M. Kissone and E. Robins, *J. Biol. Chem.* **233**, 184-8 (1958).

[37] G. Delmon, R. Babin, and P. Blanquet, *Bull. soc. pharm. Bordeaux* **91**, 211-3 (1953).

[38] C. F. Huebner, S. R. Ames, and E. C. Bubl, *J. Am. Chem. Soc.* **68**, 1621-8 (1946); L. A. Manson and J. A. Lampen, *J. Biol. Chem.* **191**, 95-104 (1951); J. T. Edward and D. M. Waldron, *Nature* **170**, 461-2 (1952); F. B. Cramer and G. A. Neville, *J. Franklin Inst.* **256**, 379-81 (1953).

[39] S. Patton and G. W. Kurtz, *J. Dairy Sci.* **34**, 669-74 (1951); W. G. Jennings, W. L. Dunkley, and H. G. Rieber, *Food Research* **20**, 13-22 (1955).

Procedure—*By 3,5-diaminobenzoic acid.* Place 0.01-1 mg. of frozen dried tissue section or 0.0025 ml. of brain homogenate in ice water. Add 0.005 ml. of 5% trichloroacetic acid solution to the tissue section or 0.0025 ml. of 9.8% trichloroacetic acid solution to the homogenate. Add 0.005 ml. of 5% trichloroacetic acid to the control. Centrifuge for 5 to 10 minutes. Discard the supernatant liquid or save it for lipid determination. Extract the lipids remaining in the sample with 0.01 ml. of 1% potassium acetate solution in 95% ethanol. Let stand for 5 to 10 minutes and centrifuge. Remove the supernatant liquid and extract the sample twice with 0.01-ml. portions of absolute ethanol. Cap the tubes, heat at 60° for 15 minutes, and centrifuge after each extraction. Dry in a centrifugal desiccator.

Dissolve 0.4 gram of 3,5-diaminobenzoic acid in 1 ml. of water. Decolorize the brown solution with 10-20 mg. of Norite by successive extractions and centrifuging. Protect the reagent from light. Alternatively, dissolve 0.3 gram of pure 3,5-diaminobenzoic acid in 1 ml. of 1:2 hydrochloric acid and decolorize with Norite.

To a sample containing 50 to 6400 millimicrograms of desoxyribosenucleic acid, add 0.001 ml. of reagent. Mix vigorously, cap tightly, and heat at 60° for 30 minutes. Add 0.001 ml. of 1:8 ammonium hydroxide solution to the blank and prepare standards containing 10 to 80 millimicrograms of desoxyribosenucleic acid. Add 0.02 ml. of 6% perchloric acid solution, mix, and add a 0.016-ml. aliquot of the solution to 0.035 ml. of 6% perchloric acid solution. Read at 520 mμ.

By diphenylamine. To an aliquot containing desoxypentose or desoxyribonucleic acid in 5% trichloroacetic acid solution, add 2 volumes of diphenylamine reagent prepared by dissolving 1 gram of diphenylamine in 98 ml. of glacial acetic acid and 2 ml. of concentrated sulfuric acid. Reflux for 10 minutes at 100°, cool in ice, and read at 650 mμ.

By periodate and thiobarbituric acid. Mix 3.5 ml. of aqueous solution containing 0.0002-0.0125 mg. of desoxyribose with 0.5 ml. of 0.5% solution of periodic acid in 1:280 sulfuric acid and mix. After 20 minutes at room temperature shake with 1 ml. of 2% sodium arsenite solution in 1:20 hydrochloric acid. After 2 minutes mix an aliquot of 1 ml. or less with 2 ml. of 0.6% thiobarbituric acid which has been adjusted to pH 2. Dilute to 3 ml. and heat at 100° for 20 minutes. Cool and read at 532 mμ correcting for a reagent blank.

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[40] V. S. Waravdekar and L. D. Saslaw, *Biochim. et Biophys. Acta* **24**, 439 (1957).

Arabinose

The familiar anthrone method for hexoses as applied to arabinose has been given in one form as applied to ribose, arabinose, and other compounds on page 175. Another modification given here applies to other sugars listed in the procedure. Careful control of time and temperature is essential because of significant lack of stability of both the reagent solutions and the developed color. Samples are added to the reagent since methylpentoses and ketohexoses develop appreciable amounts of color with the reagent at room temperature and this order of addition minimizes color development prior to heating.[41]

Sugars mentioned above exhibit their principal maxima near 625 mμ except arabinose and rhamnose. If the heating time is increased, the rhamnose maximum shifts to 610 mμ. In general at lower temperatures, the absorption maximum of monosaccharides diminishes and requires a longer development time. Exceptions are rhamnose and fructose which increase at 90°, and fructose and sorbose which increase at 60°.

The wave length of the maximum is independent of the temperature at which the color is developed.

Development of furfural with orcinol (Vol. III, pp. 189-90) is applicable to arabinose and galactose (Vol. III, p. 213) in soy beans with interference by uronic acids.[42] When glucose, galactose, lactose, xylose, and arabinose are separated on a paper chromatogram they are developed on the paper with 9.3% diphenylamine—9.6% citric acid in 50% ethanol. Thereafter the eluate of the spots is read at 420 mμ.[43]

For determination of arabinose by thymol see page 197. For determination by aminodiphenyl see page 168. For determination by phenol in sulfuric acid see page 167. For determination by orcinol see page 170. For determination of arabinose in 79% sulfuric acid see page 168. For determination by *N*-ethylcarbazole see page 168.

Procedure—*By anthrone.* This technic is applicable to glucose, galactose, rhamnose, fucose, fructose, and sorbose as well as to arabinose.

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[41] J. R. Helbert and K. D. Brown, *Anal. Chem.* 27, 1791-6 (1955); Cf. Kenjiro Yamomoto, *Nippon Yakurigaku Zasshi* 52, 602-14 (1956).

[42] S. Kawamura, T. Kobayashi, and M. Ohsima, *Kagawa Kenritsu Noka Daigaku Gakujutsu Hôkoku* 6, 241-2 (1955).

[43] J. W. Date, *Scand. J. Clin. and Lab. Invest.* 10, 149-54 (1958)

Dissolve the sample in 2:1 sulfuric acid to contain 0.05-0.1 mg. of the sugar being determined per ml. When used the solution, including time to dissolve the sample, should be 30-60 minutes old. The reagent is preferably 1-2 hours old but may be stored in the dark.

Add a 2-ml. aliquot of sample to 4 ml. of 0.16% anthrone solution in 2:1 sulfuric acid. If an aliquot of less than 2 ml. is taken, dilute the final solution to 6 ml. with 2:1 sulfuric acid. Heat for the time and temperature shown in the following schedule to develop the optimum absorption.

Monosaccharide	*Temperature*	*Duration of heating in minutes*
Glucose	90	16
Galactose	90	8
Arabinose	80	4
Rhamnose	70	13
Fucose	70	13
Fructose	60	7
Sorbose	60	8

Control the heating time to ±2 seconds. Cool in cold water for 3 minutes. Allow to rise to room temperature for 20-25 minutes and read within 30 minutes at 625 mμ. Protect the color at all times from direct sunlight.

Arabinose, ribose, glucuronolactone, galacturonic acid, xylose, fructose, galactose, glucose, mannose, or sucrose in 90% sulfuric acid.[44] Thoroughly chill 6 ml. of concentrated sulfuric acid. Layer 1 ml. of solution containing up to 0.1 mg. of a single carbohydrate on the acid and mix while still in the ice bath. Heat the solution in 90% sulfuric acid for 5 minutes at 100°, or for 30 minutes for glucuronolactone, and cool rapidly under cold water. Read at 287 mμ for arabinose and ribose, 295 mμ for glucuronolactone, 301 mμ for galacturonic acid, 316 mμ for xylose, and 322 mμ for fructose, galactose, glucose, mannose, and sucrose.

PENTOSANS

The pentosans are related to the pentoses in yielding the latter on hydrolysis. By distillation from acid solution they are converted to furfural and methyl furfural. Although substantial amounts of pentosans are determinable titrametrically, the sensitivity of this determination on highly purified pulps, containing less than 1% of pentosans, is improved by reading in the ultraviolet.[45] The titrametric method with aniline has also been developed photometrically.[46]

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[44] I. H. Bath, *Analyst* 83, 451-5 (1958).

Procedure—*In the ultraviolet.* Distil a 1-gram sample of pulp with 150 ml. of 1:7 hydrochloric acid at the rate of 1.5 ml. per minute, adding additional 1:7 hydrochloric acid during the distillation. Collect 200 ml. of distillate. Dilute an aliquot of the distillate. For pulps containing 0.5-2% of pentosans 1:10 dilution is appropriate. Read at 277.5 mμ against distilled water. While the distillate contains both furfural and methyl furfural, interpret from a furfural curve. A correction is subtracted to allow for this and therefore assumes that the ratio of furfural to methylfurfural is constant. The theoretical factor for converting furfural to pentosans is 0.727.

$$\% \text{ pentosans} = \frac{\text{grams of furfural} - 0.002}{\text{sample} \times 0.727} \times 100.$$

By aniline. Distil a 1-gram sample of pulp with 150 ml. of 1:2 hydrochloric acid at the rate of 6 ml. per minute, progressively replacing the acid distilled, until 360 ml. has gone over. Dilute a 1-25 ml. aliquot of the distillate containing pentosans which have been distilled as furfural to 30 ml. Neutralize with 24% sodium hydroxide solution using phenolphthalein indicator. Add 10 ml. of 20% sodium chloride solution, 5 ml. of 5% opalic acid solution, and 5 ml. of 10% sodium dihydrogen phosphate solution. Acidify with 20 ml. of glacial acetic acid and add 3 ml. of freshly distilled aniline. Dilute to 100 ml. and maintain at 25° for 55-60 minutes. Read at 515 mμ.

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[45] E. D. Smith and L. N. Rogers, *Anal. Chem.* 25, 931-3 (1953).

[46] R. A. Stillings and B. L. Browning, *Ind. Eng. Chem., Anal. Ed.* 12, 499-502 (1940); G. A. Adams and A. E. Castagne, *Can. J. Research* 26B, 314-24 (1948); A. S. O'Brien and T. L. Davis, *Tappi* 35, 499-505 (1952); Cf. Tappi method T223m-48.

CHAPTER 8

HEXOSES AND HEPTOSES

MANY of the hexoses are determined by the same reagents as other sugars. Some of these reagents appeared with discussions under pentoses. And, in general, the reactions discussed in this chapter, particularly under glucose, are applicable to other sugars within the chapter and to the determination of pentoses and polysaccharides. A general method for hexoses by carbazole is given on page 197. A general method for all water-soluble carbohydrates by thymol appears on page 197. A method for polyols by oxidation with bichromate in acid solution and reading the chromic ion at 610 mμ is applicable to glucose, page 42.

For determination of glucoheptose by thymol see page 197. For determination of glucuronolactone in 90% sulfuric acid see page 179. The reaction of glucose-1-phosphate with anthrone is substantially identical with that for glucose, see page 194. The same applies to glucose-6-phosphate. For determination 2-*O*-methyl-*d*-glucose, 3-*O*-methyl-*d*-glucose, or 6-*O*-methyl-*d*-glucose by aminodiphenyl see page 168.

For determination of 2,3-di-*O*-methyl-*d*-glucose, 2,3,6-tri-*O*-methyl-*d*-glucose, or 2,3,4,6-tetra-*O*-methyl-*d*-glucose by phenol in sulfuric acid see page 167. For determination of the latter by aminodiphenyl see page 168. For determination of glucurone by the color in 79% sulfuric acid see page 168.

For determination of invert sugar by reduction of ferric ion see page 201. The reaction of fructose-1-phosphate, fructose-6-phosphate, or fructose-1,6-diphosphate with anthrone is substantially identical with that for fructose, see page 201.

For determination of 2,3-di-*O*-methyl-*d*-mannose or 2,3,6-tri-*O*-methyl-*d*-mannose by phenol in sulfuric acid see page 167. For determination of galactoheptose by thymol see page 197. For determination of galacturonic acid in 90% sulfuric acid see page 179. For determination of 2,3,4,6-tetra-*O*-methyl-*d*-galactose by phenol in sulfuric acid see page 167.

For development of mannose (Cf. Vol. III, pp. 212-3 by periodic acid consumption see page 174. For determination by chromotropic acid see page 200. For determination by thymol see page 197. For determination by phenol in sulfuric acid see page 167. For determination by chromotropic acid see page 196. For determination in 90% sulfuric acid see page 179. For determination of mannose by the color in 87% sulfuric acid see page 161. For determination by carbazole in sulfuric acid see page 197. For determination by *N-ethylcarbazole* see page 168.

For determination of rhamnose by anthrone in the presence of some other sugars see pages 179 and 195. For determination by thymol see page 197. For determination by phenol in sulfuric acid see page 167. For determination by aminodiphenyl see page 168. For determination by the color in 79% sulfuric acid see page 168.

For determination of sorbose by thymol see page 197. For determination by sodium chlorite see page 200. For determination by anthrone see page 179. For determination by consumption of periodic acid see page 174. For determination by phosphomolybdic acid see page 207. For determination by the color in 79% sulfuric acid see page 168. For determination by *N*-ethylcarbazole see page 168.

For determination of maltose by phenol in sulfuric acid see page 167. For determination by aminodiphenyl see page 168. For determination by consumption of periodic acid see page 174. For determination by chromotropic acid see page 200. For determination by reduction of ferric ion see page 201. For determination by the color in 79% sulfuric acid see page 168. For determination by *N*-ethylcarbazole see page 168.

HEXOSES IN GENERAL

One of the reactions for hexoses which is generally applicable is that with carbazole in sulfuric acid.[1] Glucose, galactose, fructose, and mannose do not differ sufficiently to permit differentiation.

Procedure—Add 10 ml. of 1% carbazole in absolute ethanol to 300 ml. of 84% sulfuric acid. Chill 9 ml. of reagent in ice. Add 1 ml. of sample solution, mix, and heat at 100° for 15 minutes. Chill at once in ice and read.

ALDOSES

The usual iodometric procedure for aldoses is modified by treating the sample in alkaline solution with an excess of iodine-potassium iodide solution and measuring the excess iodine spectrophotometrically with glucose as the reference solution.[2] The amount of aldose present is determined by comparing the difference between the color of the iodine in the blank and the sample with the difference between the blank and the glucose. Sodium carbonate is used to provide the optimum pH of 11.3. In this method the ratio of iodine to aldose is greater than in other procedures.

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[1] G. Holzman, R. V. MacAllister, and C. Niemann, *J. Biol. Chem.* 171, 27-35 (1947).

[2] G. L. Miller and A. L. Burton, *Anal. Chem.* 31, 1790-3 (1959).

The reaction time varies from 5 to 60 minutes with the sugar being tested. When the structure of the sugar is unknown a reaction time of 2 hours and a temperature of 25° should be observed for monsaccharides and disaccharides and a reaction time of 2 hours and a temperature of 13° for oligosaccharides to eliminate effect of secondary reactions involving alcoholic groupings. Although the maximum absorbance is at 352 mμ, aldoses containing the equivalent of up to 2 mg. of glucose are read at 480 mμ for a more practical range of spectrophotometric readings, aldoses containing the equivalent of up to 0.2 mg. of glucose at 404 mμ and enzymatic digests of carboxylmethylcellulose at 590 mμ.

As applied to blood and urine the aldoses are reacted with *o*-toluidine in acetic acid.[3] Not more than 10% of water is permissible.

Procedure—*By iodine. Aldoses in the range of 0-2 mg. of glucose.* Prepare a 2-ml. aliquot of the sample containing not more than the equivalent of 2 mg. of glucose on a molar basis, 2 ml. of glucose solution containing 2 mg. of glucose, and a 2-ml. water blank. To each tube add 1 ml. of 8% sodium carbonate solution and 2 ml. of iodine-potassium iodide solution containing 0.26% of iodine and 10% of potassium iodide. The reagent need not be standardized. Mix by swirling and let stand for an amount of time based on Table 13. Acidify with 4 ml. of 1:35 phosphoric acid and mix with a pumping motion with a 3-mm. glass rod having a 7-mm. flattened end to prevent too rapid an evolution of carbon dioxide,

TABLE 13. REACTION TIME FOR ALDOSES WITH IODINE

Aldose	*Rate of reaction with iodine at 25°*
Galactose	5 minutes
Glucose	15 minutes
Cellobiose	20 minutes
Cellopentaose	60 minutes
Carboxymethylcellulose digest	35 minutes
Mannose	60 minutes

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[3] J. Ek and E. Hultman, *Nature* 181, 780-1 (1958); Eric Hultman, *Ibid.* 183, 108-9 (1959).

to remove the excess carbon dioxide and to avoid error due to the formation of gas bubbles in the spectrophotometric cuvets. Read at 480 mμ.

Aldoses in the range of 0-0.2 mg. of glucose. Follow the procedure for aldoses in the range of 0-2 mg. of glucose, substituting the appropriate glucose standard. The reagent contains 0.026% of iodine and 10% of potassium iodide. Double the reaction time based on Table 13 and read at 404 mμ.

Enzymatic digests of carboxymethylcellulose. Incubate 1 ml. of buffered carboxymethylcellulose and 1 ml. of cellulase containing 4 units of enzyme activity at 50° for 80 minutes and cool rapidly. Follow the procedure for aldoses in the range of 0-2 mg. glucose, and read at 590 mμ after 35 minutes.

By o-*toluidine. Blood.* Mix 1 ml. with 1 ml. of 3% trichloroacetic acid. Centrifuge and mix 0.2 ml. of the clear centrifugate with 3 ml. of 6% *o*-toluidine in glacial acetic acid. After 8 minutes at 100° cool and read at 625 mμ against a reagent blank.

Urine. Boil 0.004 ml. with 3 ml. of 2% *o*-toluidine in glacial acetic acid for 8 minutes. Cool and read at 625 mμ against a reagent blank.

GLUCOSE, DEXTROSE

Many modifications of the anthrone method (Vol. III, p. 207) for determining hexoses have been introduced [4] to eliminate errors and reduce interferences from pentoses. A device for the controlling of mixing of the reagent with the sample reduces errors caused by premature or irregular mixing. A small conical flask mounted in a spinning disk is one means of keeping reactants in separate layers.[5] The reagent is added down the side of the rotating flask and collects at the bottom. The flask is then removed from the disk and shaken. A specific method is applicable to deproteinized tissue extract.[6] Details of application of anthrone to pentoses have already been given on page 178.

In the presence of pentoses, the complex formed with anthrone is of a different nature than that formed when hexoses are anthronated alone.[7] Therefore subtraction of a correction based on a separate determination of the pentose is not feasible. Replacement of concentrated sulfuric acid by an 81% concentration reduces pentose errors from 50% to 10%. Since the wave length of maximum absorbance is not altered from 625 mμ the reaction is probably the same. Sulfuric acid prepared by mixing 72:18 with water approximates this dilution.[8] Maximum color development at 100° then varies as to time, glucose in 12 minutes but sucrose in 3-8 minutes and fructose in 1.5 minutes. Too low an acid concentration reduces the sensitivity of the method. The ratio of pentose to hexose is also a determining factor in pentose interference, the greatest interference occurring when the pentose:hexose molecular ratio is 2.7:1. With a pentose ratio of 3:1 the maximum absorption shifts from 625 mμ to 680 mμ.

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[4] R. E. Zipf and A. L. Waldo, *J. Lab. Clin. Med.* 39, 497-502 (1952); P. Confortini and A. Dagradi, *Patol. sper. chir.* (*Milan*) 1, 708-17 (1953); N. Uezumi, S. Hasegawa, and K. Kaneko, *Siekagaku* 29, 51-4 (1957).

[5] Ronould Johanson, *Anal. Chem.* 26, 1331-3 (1954).

[6] John Paul, *Analyst* 83, 37-42 (1958).

The fact that various carbohydrate classes can be identified by the time required for each to reach maximal color development by controlled heating with the anthrone reagent serves as a basis for the simultaneous determination of sugars.[9] The color by this technic reaches a maximum for glucose in between 30 and 40 minutes but glucose and its phosphates are estimated at 25 minutes. Other aldohexoses behave like glucose but generally give less color. Sorbose behaves like fructose and gives 83% of the color of fructose.

The 6-desoxyhexoses, rhamnose and fucose, exhibit maximal color intermediate between that of aldohexoses and aldopentoses with respect to time. Adonitol, ascorbic acid, glucoheptonate, galactonolactone, glucuronolactone, and mucic acid give no more than 4% of the color of glucose. The reproducibility and precision of the method is increased when the reaction mixture is heated in an 80° bath in preference to a 100° bath. The color intensity peaks are broader and a longer time is allowed at temperature equilibrium at 80°. Fructose, glucose, and their phosphates are determined simultaneously by this method.

The following molarities of ions do not interfere: 1 sodium, 1 potassium, 0.01 barium, 0.01 calcium, 1 ammonium, 0.1 magnesium, 0.05 manganese, 1 cyclohexylammonium, 0.1 chloride, 0.05 borate, 1 phosphate, 0.5 trichloracetate, 0.1 chlorate, and 0.1 formate. The following molarities of ions interferes strongly: 0.2 nitrite and 0.001 azide. Lactic acid and glycerol do not react. Uronic acids give a small reaction.[10]

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[7] R. Johanson, *Nature* **171,** 176-7 (1953).

[8] N. J. Fairbairn, *Chem. & Ind.* (*London*) **1953,** 86.

[9] L. H. Koehler, *Anal. Chem.* **24,** 1576-9 (1952); Lewis C. Mokrasch, *J. Biol. Chem.* **208,** 5-9 (1954); Cf. S. J. Provhovnik and J. F. Nelson, *Australian J. Exptl. Biol. Med.* **31,** 279-82 (1953); S. L. Bonting and S. Bonting, *Arch. Biochem. and Biophys.* **52,** 272-9 (1954).

Creatinine, glutathione, ascorbic acid, cystine, glycine, leucine, tryptophan, aspartic acid, albumin, lactic acid and phenol do not interfere if the sample is deproteinized with cadmium hydroxide.[11]

When glucose, fructose, or related carbohydrates are heated with concentrated sulfuric acid (Vol. III, p. 206), a bluish-pink color develops, which is specific and dependent upon the acid concentration.[12] The color does not depend on the reducing power of the sugar, but rather on the formation of 5-(hydroxymethyl) furfural by dehydration of glucose. Pentoses are incapable of forming formaldehyde under these conditions and do not interfere. Beer's law is followed but the yield of formaldehyde produced is not quantitative when calculated on a molar basis as this reaction is probably only one alternative pathway for the decomposition of hexoses in strong acid.

As an extension of this reaction, chromotropic acid in sulfuric acid reacts with glucose, galactose, mannose, and fructose to produce a violet color. This reaction depends on the conversion of hexoses to 5-hydroxymethylfurfural and the splitting of the methylol group to form formaldehyde which reacts with the chromotropic acid to form the violet color.[13] Lactose, sucrose, and maltose also react but arabinose, xylose, and rhamnose do not. The reaction is not quantitative and therefore conditions of development require careful control. Glucose, for example, gives only about a 30% yield of formaldehyde. The yield varies for other sugars.

Fehling's solution is reduced by sugar to cuprous oxide which can then be oxidized to cupric oxide for weighing. A time-saving alternative is to react the copper compound with ethylenediamine to form a copper ethylenediamine complex which is read at 550 mμ[14] (Cf. Vol. III, p. 206). Addition of phloroglucinol increases the coloration and eliminates interference caused in deproteinizing biological samples.[15] A standard which is stable for 6 months for blood glucose by this method consists of cobalt chloride in 60 grams of glycerol containing 2 ml. of water.[16] Residual color after reduction of cerebrospinal fluid by Fehling's solution is an alternative to reading the color obtained by solution of the precipitated copper oxide in acid [17] (Vol. III, p. 206).

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[10] E. W. Yenim and A. J. Willis, *Biochem. J.* **57**, 508-14 (1954).

[11] Itsuro Takashita, *Igaku to Seibutsugaku* 34, 130-4 (1955).

[12] B. Mendel and P. L. Hoogland, *Lancet* 259, 16-7 (1950); B. Mendel, A. Kemp, and D. K. Myers, *Biochem. J.* 56, 639-46 (1954).

[13] B. Klein and M. Weissman, *Anal. Chem.* 25, 771-4 (1953).

[14] M. E. Hobbs and F. L. Layton, *Ibid.* 26, 585-6 (1954).

[15] R. S. Martin, *Chemist Analyst* 45, 73-5 (1956).

[16] A. Kemp and M. Zuidweig, *Acta Physiol. Pharmacol. Neerland* 2, 280-5 (1951-52).

In the presence of strong sulfuric acid different sugars give characteristic absorption curves with thymol.[18] Since any protein present has only a slight effect on the absorption curve, due to the reaction of the sulfuric acid with the protein, sugars bound to proteins are determined by this method. Glycosidic linkages do not interfere. Beer's law is followed for 0.01-0.1 mg. of sugar in the sample.

The interaction of glucose with phenylhydrazine produces glucosazone. the intensity of its yellow color being proportional to the concentration of glucose. The color reaction is specific for ketoses and aldoses and the color is stable.[19] Noncarbohydrate substances such as sodium chloride, uric acid, urea, creatine, creatinine, glutathione, methionine, tryptophan, and tyrosine, do not form a yellow color with the reagent. Ascorbic acid, trichloroacetic acid, metaphosphoric acid, and sodium tungstate interfere. Because of this, zinc acetate is used as the deproteinizing agent in blood samples prior to glucose determination by phenylhydrazine. Addition of anticoagulants has no effect on glucose determination. Since the phenylhydrazine method for determining glucose is not based on the reducing power of glucose it is possible to determine the true glucose content in blood samples containing other reducing agents.

Another reagent for glucose estimation is phenol and sulfuric acid.[20] The sensitivity of this method is nearly the same as the anthrone method but it has the advantages that the phenol reagent is stable for a long period, the method is not affected by slight variations in the experimental conditions, and the effect of coexisting impurities is less. Most amino acids, higher alcohols, and organic acids do not interfere, while proteins increase the extinction values. Beer's law is followed up to 0.005% of sugar and calibration curves permit the determination of up to 0.01% of hexose. Details of its application appear on page 167.

Glucose, galactose, mannose, glucuronic acid, and fucose as well as mixtures of these sugars are determined by carbazole.[21] Sugars, bound or in free form, give the same results with carbazole reagent except in cases where there is a loss of sugar from interaction with proteins or with mineral acid. Noncarbohydrate components do not interfere.

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[17] F. Ragazzini and R. Cocchi, *Riv. clin. pediat.* 50, 608-16 (1952); Cf. F. Moya and J. Dewar, *J. Lab. Clin. Med.* 47, 314-9 (1956).

[18] M. R. Shetlar and Y. F. Masters, *Anal. Chem.* 29, 402-5 (1957); Cf. J. Martinez Bruna, *Diagn. biol.* 3, No. 1, 21-8 (1954).

[19] N. Wahba, S. Hanna, and M. M. El-Sadr, *Analyst* 81, 430-2 (1956).

[20] Otto Balazs, *Magyar Kém. Folyóirat* 59, 70-7 (1953); M. Duboís, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.* 28, 350-6 (1956); Y. Matsuo and A. Nanba, *Bull. Fac. Engr. Hiroshima Univ.* 5, 19-24 (1956); Cf. J. L. Keen and J. W. Opie, *Tappi* 40, 100-4 (1957).

[21] R. E. Glegg, *Anal. Chem.* 28, 532-4 (1956).

Reduction of picric acid to picramic acid by glucose in alkaline solution (Vol. III, p. 206) is applicable to deproteinized blood [22] or to urine.[23]

Glucose and fructose are determined simultaneously by 2,4-dinitrophenylhydrazine. The sample is heated with 1:1.4 hydrochloric acid saturated with 2,4-dinitrophenylhydrazine. The uncoupled reagent is destroyed with 11% potassium hydroxide solution and ethanol is added. The hydrazones dissolve as their highly colored quinoid form. In protein-free mixtures up to 0.002 mg. of fructose and up to 0.01 mg. of glucose per ml. can be so determined.[24]

Up to 0.15 mg. each of both glucose and galactose can be determined simultaneously by orcinol. The reagents should be kept in an ice bath to stabilize results by preventing color formation prior to heating. Ribose gives the same color with the reagent.[25] When the orcinol reaction is applied to protein-bound hexoses in serum the protein is precipitated by ethanol, washed, and dissolved in dilute aqueous alkali.[26] Heating with the reagent frees the hexose. Ribose and glucose are also measured together in the same solution with orcinol.[27] Sedoheptulose, fructose, and glucuronic acid do not interfere.

Glucose can be oxidized to formaldehyde by periodate.[28] Then sodium sulfite is added to reduce excess periodate and iodate formed. Aeration is necessary to reverse the reduction of the formaldehyde acid color by sulfite or sulfur dioxide. Thereafter the formaldehyde is developed with chromotropic acid. Diacetyl is converted to acetic acid and therefore does not interfere. Glycolic acid, a product of periodate scission, interferes at high sulfuric acid concentrations since the glycolic acid is oxidized by the acid to formaldehyde. A less concentrated sulfuric acid eliminates this interference. "Saccharoids" commonly present in blood do not interfere. When glucose in an acetone-free blood filtrate is oxidized to formaldehyde by periodate at room temperature, the latter is then determined by mercuric chloride in alkaline solution.[29] For determination of glucose by consumption of periodic acid see page 174.

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[22] Hans J. Wissfeld, *Die Medizinische* **1955, 712-13.**

[23] Gennero Giannettasio, *Laboratorio* **7,** 515-7 (1952).

[24] D. F. Cheesman, *J. Physiol.* (*London*) **116,** 18P-19P (1952).

[25] R. G. Hansen and Horace W. Norton, *Anal. Chem.* **27,** 857-9 (1955).

[26] M. Schönenberger, H. Kellner, H. Südhof, and H. Haupt, *Z. physiol. Chem.* **309,** 145-57 (1958).

[27] J. Dubovský, M. Honsová, F. Kalousek, J. Palek, and J. Šonka, *Chem. Listy* **52,** 1796-800 (1958).

[28] J. C. Speck, Jr., and A. A. Forist, *Anal. Chem.* **26,** 1942-4 (1954); S. L. Tompsett, *Anal. Chim. Acta* **19,** 360-3 (1958).

Since sodium chlorite oxidizes aldoses more rapidly than ketoses at pH 4, glucose is estimated in the presence of fructose by measuring the difference in chlorine dioxide produced in a definite period of time by oxidation of the sample and of a reference fructose standard.[30] Although chlorine dioxide shows an absorption maximum at 358 mμ, either the 435.8 mμ mercury line or the 436.0 mμ tungsten line should be used to avoid the absorption of sodium chlorite at 358 mμ. Correction factors are applied to compensate for loss of chlorine dioxide, probably from hydrolytic disproportionation, and to compensate for the difference in chlorite concentration in test and control solutions. Fructose, sorbose, sucrose, and raffinose react similarly, so this method may be extended to determine aldoses in the presence of ketoses.

Glucose reacts mole for mole with a solution containing *p*-anisyl tetrazoleum blue at pH 12.5 on boiling for 45 to 60 seconds. The product is the insoluble blue diformazan. This is then dissolved in dioxane for reading.[31] A minimum of 0.001 mg. and a maximum of 0.2 mg. of glucose can be estimated in a final volume of 10 ml. This is equivalent to a reducing sugar concentration of from 4 to 800 mg. in 100 ml. of blood.

Sugars on heating in aqueous alkaline solution with 2,3,5-triphenyltetrazolium chloride form a red precipitate. This is soluble in isopropanol or ethanol for reading at 490 mμ.[32] The reaction is one of reduction and therefore is obtained with glucose, fructose, cuprous oxide, etc.

Glucose gives a red color when heated with catechol in sulfuric acid at 140-5°.[33] Glycerol and some carbonyl compounds interfere and are removed by extraction with ethyl acetate. Many reducing substances do not interfere. Resorcinol is used similarly.[34]

The determination of glucose as molybdenum blue by phosphomolybdate (Vol. III, pp. 204-5) is another method depending on the reducing power of glucose. The sugar first reduces a copper solution. The color developed is directly proportional to the concentration of phosphomolybdic reagent as well as to the concentration of reduced copper and is inversely proportional to the temperature of the mixture. Changes in concentration of reagent or in temperature causes slow changes in optical density of the color until an equilibrium is reached.[35] The use of dry reagents for this reaction produces a very stable color which possibly increases slightly in intensity during the first half-hour.[36] Color fading is best prevented by use of a 1:6 phosphoric diluent although it is not entirely satisfactory.[37] Glucose condensed with aminodiphenyl gives a characteristic color.[38] For details of the application see pages 162 and 168.

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[29] M. Takada and Y. Takahashi, *Chiyro* **36**, 604-9 (1954).

[30] F. Stitt, S. Friedlander, H. J. Lewis, and F. E. Young, *Anal. Chem.* **26**, 1478-84 (1954).

[31] N. D. Cheronis and M. C. Zymaris, *Mikrochim. Acta* **6**, 769-83 (1957).

[32] Katsubiko Amako, *Seito Gijutsu Kenkyukaishi* **7**, 65-9 (1958).

[33] S. C. Harvey and V. Higby, *Texas Repts. Biol. Med.* **11**, 489-93 (1953).

[34] P. Iannella, G. Marano, P. Mazzei, and E. Scala, *Boll. soc. ital. biol. sper.* **33**, 154-7 (1957).

Glucose and fructose in body fluids are determined by condensation with other aromatic amines to give colored products in nearly water-free acid solutions. Coupling with *p*-aminobenzoic acid gives absorbances directly proportional to the sugar concentration between 0.05% and 1.2% in blood or serum and between 0.1 and 6% in urine. With *m*-aminophenol as coupling agent, absorbances are proportional to the sugar concentration between 0.05% and 0.6% of glucose in blood or serum and between 0.5 and 6% in urine.[39]

After oxidation of sugars by potassium ferricyanide the resulting ferrocyanide is estimated as Prussian blue (Vol. III, p. 204) or as molybdenum ferrocyanide. The latter is dissolved in oxalic acid in the presence of trichloroacetic acid.[40] To avoid interference samples are deproteinized with aluminum tungstate.

Glucose is determined in the presence of hexose phosphates and other hexoses except 2-desoxy-*d*-glucose by oxidation with glucose oxidase to form hydrogen peroxide which subsequently oxidizes *o*-dianisidine in the presence of peroxidase. This enzymatic reaction is carried out in a phosphate buffer at pH 5.9 and 45° for 30 minutes. The addition of sulfuric acid to a final concentration of 30% produces a stable pink color with a maximum absorption at 530 mμ.[41] As applied to determination of glucose and of sucrose in sirups the method is given on page 212.

As another variation the hydrogen peroxide formed oxidizes *o*-tolidine in the presence of horseradish peroxidase to yield a chromogen with a maximum absorbance at 365 mμ and 635 mμ.[42] The oxidized *o*-tolidine shows a broad band at 635 mμ and a narrow band at 365 mμ which has a greater sensitivity and is about 68% higher than the broad band. The sensitivity may therefore be increased by operating at 365 mμ, increasing the concentration of glucose oxidase, decreasing the final sample volume without changing the concentration of glucose oxidase, or by using a smaller amount of reagent. The lower limit of the procedure is 0.005 mg. of glucose per ml. Beer's law is followed up to 15 mg. per 100 ml. An acetate buffer at pH 4.1-4.2 is used in preference to a phosphate buffer because the latter precipitates the *o*-tolidine. The enzymes should be moisture-free and each lot of glucose oxidase should be standardized by adding graded amounts of reagent prepared from the glucose oxidase to the sample. The addition of 0.05 mg. of glucose to the sample containing suitable concentration of enzyme should produce an absorbance at 635 mμ of 0.25-0.3 unless greater or lower sensitivity is desired. The sensitivity increases with increasing enzyme concentration. This is not the case with horseradish peroxidase and standardization is not necessary.

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[35] Stanley Dische, *J. Clin. Pathol.* 8, 253-61 (1955); Dante Giarnieri, *Rass. med. sper.* 4, 364-8 (1957); Cf. D. B. Tonks, *Am. J. Clin. Pathol.* 22, 1009-17 (1952).

[36] E. Dannenberg, *Actas Bioquím.* 2, No. 7, 7-8 (1953).

[37] P. G. Higgins and H. Lehmann, *Clin. Chim. Acta* 1, 178-88 (1956).

[38] G. Athanáil and P. C. Cabaud, *J. Lab. Clin. Med.* 51, 321-4 (1958).

[39] J. Ek and E. Hultman, *Nature* 181, 780-1 (1958).

[40] I. St. Lorant, *J. Clin. Pathol.* 10, 136-7 (1957).

[41] A. Saifer and S. Gerstenfeld, *J. Lab. Clin. Med.* 51, 448-59 (1958); R. B. McComb and W. D. Yushok, *J. Franklin Inst.* 265, 417-22 (1958).

[42] L. L. Salomon and J. E. Johnson, *Anal. Chem.* 31, 453-6 (1959).

If the temperature in the room differs from 25°, the reaction mixture should be incubated at 25°. The color stability deteriorates with increasing temperature. Untreated or undiluted urine samples contain interferences which are removed by use of the ion-exchange resins Amberlite IR-120 and IR-45. Other ion-exchange resins must be tested to determine if they produce peroxides which interfere. Ascorbic acid, which is partially oxidized during treatment with the resins to form hydrogen peroxide, intensifies the color. Since the urine contains only a small amount of ascorbic acid and the dilution decreases its influence, the effect of ascorbic acid is negligible. However, if the effect of ascorbic acid must be considered, a blank containing an aliquot of the specimen and all reagents except glucose oxidase is prepared. Protein precipitation other than by zinc sulfate, oxidation with iodine and bromine followed by ether extraction, and use of adsorbant charcoal are not effective in treating urine samples. Tungstic acid forms a slightly soluble salt with the *o*-tolidine and cannot be used.

In blood samples trichloroacetic acid should be avoided as a deproteinizing agent since hydrogen peroxide is liberated. Fluoride should not be used as an inhibitor. Oxalated or heparinized blood samples are acceptable. Chlorides, thymol, and fungistatic agents interfere. Oxidizing agents on glassware should be avoided. Although *o*-tolidine sulfate gives better results, the dihydrochloride is used because of its ease of preparation, as the loss of sensitivity resulting from the dihydrochloride is not large.

If the solution is turbid, a small crystal of ascorbic acid is added or the solution is mixed with a rod dipped in a solution of ascorbic acid to bleach the color and permit the measurement of the suspended material. If the reagent blank has a considerable absorbance it should be similarly treated. In this case the absorbance of the oxidized *o*-tolidine = the absorbance of the sample − the absorbance of the blank − residual absorbance of the sample + residual absorbance of the blank.

Rochelle salt, a component of the dinitrosalicylic acid reagent for reducing sugars (Vol. III, p. 206), causes loss of glucose and interferes with the removal of oxygen by the sulfite component of the reagent.[43] However, since the Rochelle salt is essential to color stability, it may be added after color development and prior to cooling, or known amount of glucose may be added to the reducing sugar to compensate for the loss. In addition to Rochelle salt, dinitrosalicylic acid reagent contains phenol, which increases the amount of color produced and balances the effect of phenol found in urine samples, sulfite which removes the oxygen which would oxidize the glucose, and sodium hydroxide which is necessary for the reducing action of the glucose on the dinitrosalicylic acid to form 3-amino-5-nitrosalicylic acid. The same reaction is given by fructose and galactose. Disaccharides such as maltose and lactose also react but the calibration curve is different.

A yellow color is formed by the reaction of glucose with 3,4-dinitrobenzoic acid.[44] After hydrolysis of sugars, they are separated by paper chromatography. On extraction with methanol from the appropriate area of the chromatogram they are heated with aniline phthalate after evaporation of the methanol. The colored product is then read.[45] A similar technic is applicable to methylated sugars.[46]

When glucose, galactose, lactose, xylose, and arabinose are separated on a paper chromatogram they are developed on the paper with 9.3% diphenyl amine −9.6% citric acid in 50% ethanol. Thereafter the eluate of the spots is read at 420 mμ.[47]

Not only glucose but other reducing sugars are determined by reduction of ferric ion and the determination of the excess ferric ion by thiocyanate[48] (Vol. II, pp. 307-12, Vol. IIA, pp. 229-31). To illustrate the equivalents of 5 mg. of common reducing sugars in terms of mg. of iron are glucose 0.288; fructose 0.266; invert sugar 0.275; lactose 0.198; and maltose 0.173.

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[43] V. V. Kozlov and V. N. Khrustaleva, *Sbornik Nauch. Probat Moskov. Inst. Narod. Khoz. im G. V. Plekhanova* **1957**, No. 10, 352-72; Gail Lorenz Miller, *Anal. Chem.* **31**, 426-8 (1959).

[44] T. Takemoto, K. Daigo, and T. Takai, *J. Pharm. Soc. Japan* **75**, 1024 (1955).

[45] J. Blass, M. Macheboeuf, and G. Núñez, *Bull. soc. chim. biol.* **32**, 130-5 (1950).

[46] J. K. Bartlett, L. Hough, and J. K. N. Jones, *Chem. & Ind.* (*London*) **1951**, 76 (1951).

[47] J. W. Date, *Scand. J. Clin. and Lab. Invest.* **10**, 149-54 (1958).

[48] J. K. Roy, *J. Indian Chem. Soc., Ind. & News Ed.* **19**, 83-6 (1956).

For determination of glucose in 90% sulfuric acid see page 179. A method of determination in 79% sulfuric acid appears on page 168. For determination by carbazole in sulfuric acid see page 197. For determination by *N*-ethylcarbazole see page 168.

Samples—*Grass silage.*[49] Reflux a one-gram sample for 30 minutes with 100 ml. of 1:140 sulfuric acid. Filter hot, cool, and refilter. Dilute 1 ml. of the digest to 25 ml. with water and develop with anthrone. This determines all water-soluble carbohydrates.

Serum. Add a 0.5-ml. sample to 9.5 ml. of 1:9 perchloric acid, shake, and let stand for 10 minutes. Filter and determine with anthrone in sulfuric acid.[50] This gives both the free glucose and that bound to protein.

To determine only the glucose combined with protein,[51] deproteinize a 0.1-ml. sample with 4 ml. of absolute ethanol and centrifuge. Decant the upper layer. Extract the precipitate with a fresh 4-ml. portion of absolute ethanol. Centrifuge and decant. Dissolve the proteins in 3.9 ml. of physiological salt solution and develop with anthrone.

To determine the glucose combined with protein, using thymol as the reagent,[52] dilute a sample five times with 0.9% saline solution. Pipet a 0.2-ml. aliquot into 10 ml. of absolute ethanol, stopper, and mix by inversion. Wash the stopper and tube with 5 ml. of absolute ethanol. Centrifuge, wash with 10 ml. of absolute ethanol, and recentrifuge. Decant the ethanol and suspend the precipitate in 1 ml. of water for development.

Blood.[53] Deproteinize a 1-ml. sample with 5 ml. of 10% trichloroacetic acid solution. Centrifuge and treat 1 ml. of the supernatant liquid with 0.05 ml. of a stannous chloride solution prepared by dissolving 1 part of stannous chloride in 10 parts of concentrated hydrochloric acid and diluting the mixture with 9 volumes of water before use. Add 3 ml. of concentrated sulfuric acid and let stand for 5 minutes at 30°. Develop with anthrone.

Alternatively,[54] for development with anthrone, mix a 0.1-ml. sample with 2 ml. of cadmium hydroxide solution containing 26 grams of cadmium hydroxide and 127 ml. of 1:35 sulfuric acid per liter. Add 0.5 ml. of 4.4%

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[49] A. John, G. Barnett, and T. B. Miller, *J. Sci. Food Agr.* **1**, 336-9 (1950).

[50] V. Kapuscinski and B. Zak, *Am. J. Clin. Pathol.* **23**, 784-8 (1953).

[51] C. v. Holt, *Klin. Wochschr.* **32**, 661-2 (1954).

[52] M. R. Shetlar and Y. F. Masters, *Anal. Chem.* **29**, 402-5 (1957).

[53] Koki Motegi, *Nisshin Igaku* **37**, 95-7 (1950).

[54] Itsuro Takashita, *Igaku to Seibutsugaku* **34**, 130-4 (1955).

sodium hydroxide solution and heat for 3 minutes at 100°. Cool and centrifuge.

For development with sulfuric acid,[55] add a 0.1-ml. sample to 1.9 ml. of a deproteinizing solution consisting of 5% trichloroacetic acid solution containing 0.1% of silver sulfate. The latter removes chloride. Centrifuge for 5 minutes and develop an aliquot of the clear layer.

As an alternative method of deproteinizing for development with sulfuric acid,[56] to a 0.05-ml. sample, add 1.85 ml. of an isotonic solution prepared by dissolving 9.6 grams of sodium sulfate decahydrate and 2.1 grams of copper sulfate pentahydrate in 100 ml. of water. Add 1 ml. of 10% sodium tungstate solution, mix, and centrifuge to remove the protein. Develop a 1-ml. aliquot.

For development with hydrazine, add a 1-ml. sample to 9 ml. of 10% zinc acetate solution. Mix and heat for 3 minutes at 100°. Filter and develop an aliquot of the filtrate.

For development as molybdenum blue,[57] add a 0.1-ml. sample to 4.6 ml. of an isotonic sodium sulfate-copper sulfate solution, prepared by mixing 320 ml. of 3% sodium sulfate decahydrate solution with 30 ml. of 7% copper sulfate pentahydrate solution. Add 0.3 ml. of 10% sodium tungstate solution, mix, and centrifuge to remove protein.

Protein.[58] Triturate a 2-gram moisture-rich sample or a 0.3-gram moisture-free sample with 20 ml. of acetone for 30 minutes. Filter the liquid and digest the residue at 100° with two 20-ml. portions of acetone. Evaporate the combined solvents, add water, and extract with petroleum ether. Reflux the original residue combined with the residue from the petroleum ether extraction with 10 ml. of 4% sodium hydroxide solution until dissolved, for a maximum of 30 minutes. Dilute to 100 ml. with water. Develop with anthrone.

Technics for development of the original sample with picric acid or with mercuric chloride appear under procedure.

Procedure—*By anthrone.*[59] *Pentoses present.* As a reagent, dissolve 100 mg. of anthrone in 100 ml. of 81% sulfuric acid. Let stand for 12 hours.

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[55] Nicoline Russo, *Il Progr. med.* **8**, 272-5 (1952); B. Mendel, A. Kemp, and D. K. Myers, *Biochem. J.* **56**, 639-46 (1954).

[56] R. S. Martin, *Chemist Analyst* **45**, 73-5 (1956).

[57] Stanley Dische, *J. Clin. Pathol.* **8**, 253-61 (1955).

[58] C. H. Brieskorn and H. W. Berg, *Z. Lebensm.-Untersuch. u. -Forsch.* **108**, 170-4 (1958).

[59] Ronould Johanson, *Anal. Chem.* **26**, 1331-3 (1954).

Cause a 30-ml. conical flask with a 3-ml. aliquot containing 0.03-0.18 mg. of glucose to rotate upright at 400 rpm as held on a disk. Add 9 ml. of reagent rapidly down the side wall. Remove the flask from the disk and mix immediately by shaking. Heat for 7 minutes at 90°. Cool and let stand for 30 minutes. Read at 625 mμ.

Simultaneous determinations of glucose and another sugar.[60] As a reagent mix 1 liter of concentrated sulfuric acid with 290 grams of ice. After the solution has cooled, add 1 gram of anthrone. Stored at 4° in the dark it is usable for months.

Chill 6 ml. of reagent in an ice bath for 5 minutes. Add 1 ml. of sample containing up to 0.25 micromole of ketohexose, up to 0.4 micromole of aldohexose, or up to 1 micromole of pentose without mixing. Chill, mix rapidly by swirling, and return to the ice bath. Cover and heat at 80° for a definite period of time depending on the sugar. Return to the ice bath and read at 620 mμ. Samples may be stored in the ice bath for several hours before reading.

Appropriate periods of heating in minutes are: fructose 3, fructose-1-phosphate 3, fructose-6-phosphate 3, fructose-1,6-diphosphate 3, glucose 25, glucose-1-phosphate 25, glucose-6-phosphate 25, rhamnose 8, fucose 8, ribose 5, adenosine 5. For raffinose, melezitose, sucrose, and turanose the optical density is approximately constant after 20 minutes heating.

By reading after heating for different time intervals binary mixtures are calculated by simultaneous equations. For glucose and fructose the following calculation is illustrative.

$$C_F = \frac{D_3 - k_G D_{25}}{E_F\,(1 - k_F k_G)}$$

$$C_G = \frac{D_{25} - k_F D_3}{E_G\,(1 - k_F k_G)}$$

where

C_F is micromoles of fructose in the 1-ml. sample

C_G is micromoles of glucose in the 1-ml. sample

E_F and E_G are the optical densities obtained from 1 micromole of fructose and glucose at 3 minutes and 25 minutes, respectively

D_3 and D_{25} are the densities of tubes after heating for 3 minutes and 25 minutes respectively

k_G equals D_3/D_{25} for glucose and

k_F equals D_{25}/D_3 for fructose.

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[60] Lewis C. Mokrasch, *J. Biol. Chem.* 208, 55-9 (1954).

Tissue extract.[61] Add a 1-ml. aliquot containing 0.01-0.15 mg. of glucose or its equivalent to a 5-ml. portion of 0.2% anthrone solution in 2:1 sulfuric acid so that the sample forms a layer above the reagent. Mix with a glass rod with a flattened end by plunging the rod up and down in the mixture once or twice. Heat immediately at 100° for 10 minutes and cool in ice. Read the green color at 620 mμ.

By concentrated sulfuric acid.[62] Add 1 ml. of protein-free sample containing up to 0.15 mg. of glucose to 3 ml. of concentrated sulfuric acid. Heat at 100° for exactly 6.5 minutes. Cool and read at 520 mμ.

Alternatively,[63] add 1 ml. of protein-free sample containing up to 0.8 mg. of glucose to 100 ml. of concentrated sulfuric acid. Mix and heat at 37° for 7 minutes. Cool and read at 450 mμ.

By chromotropic acid. Sulfuric acid oxidation.[64] As a reagent, dissolve 100 mg. of sodium 1,8-dihydroxynaphthalene-3,4-disulfonate in 1 ml. of water and dilute to 50 ml. with 15 *M* (6:1) sulfuric acid. Prepare fresh daily.

Add 5 ml. of reagent to 1 ml. of protein-free sample containing 0.05-0.3 mg. of hexose and heat for 30 minutes at 100°. Cool to room temperature and dilute to 10 ml. with 1:1 sulfuric acid. Read at 570 mμ. This reaction is given by glucose, galactose, mannose, fructose, lactose, sucrose, and maltose.

By copper and ethylene diamine.[65] Mix an aliquot of sample containing over 50 mg. of hexose and no other reducing substance with an excess of Fehling's solution. Heat in boiling water until reaction appears to be complete. Centrifuge to separate the cuprous oxide and decant. Wash the precipitate with 5 ml. of water, centrifuge, and decant.

Dissolve the cuprous oxide precipitated in 75 ml. of hot 1:3 nitric acid by slow addition of the acid. Dilute to 200 ml. and mix. To a 10-ml. aliquot add 10 ml. of 6% ethylenediamine solution. Dilute to 50 ml. and mix. Read the copper ethylenediamine complex at 550 mμ.

By phenylhydrazine.[66] To prepare the reagent, dissolve 1 gram of phenylhydrazine hydrochloride in 90 ml. of warm water and add 5 ml. of a saturated sodium metabisulfite solution. Dilute to 100 ml. with water and store in a dark bottle.

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[61] John Paul, *Analyst* **83,** 37-42 (1958).

[62] B. Mendel and P. L. Hoogland, *Lancet* **259,** 16-7 (1950); B. Mendel, A. Kemp, and D. K. Myers, *Biochem. J.* **56,** 639-46 (1954).

[63] R. S. Martin, *Chemist Analyst* **45,** 73-5 (1956).

[64] B. Klein and M. Weissman, *Anal. Chem.* **25,** 771-4 (1953).

[65] M. E. Hobbs and F. L. Layton, *Ibid.* **26,** 585-6 (1954).

[66] N. Wahba, S. Hanna, and M. M. El-Sadr, *Analyst* **81,** 430-2 (1956).

To a 5-ml. protein-free sample containing 0.5-2 mg. of glucose, add 2 ml. of phenylhydrazine reagent and 3 ml. of glacial acetic acid. Deproteinizing with trichloroacetic acid, metaphosphoric acid, or sodium tungstate is not permissible due to interferences. Zinc acetate is suitable. Heat for 1 hour at 100° and let cool to room temperature. Dilute to 10 ml. and read at 435 mμ.

By carbazole.[67] Purify the carbazole by dissolving it in 84% sulfuric acid, precipitating with water, and recrystallizing from ethanol and then from toluene. A 0.5% carbazole solution in absolute ethanol should be colorless when mixed with 84% sulfuric acid. The concentration of the acid should have been checked by titration.

Prepare reagent *A* by diluting 8.35 ml. of absolute ethanol to 250 ml. with 84% sulfuric acid and reagent *B* by diluting a 0.5% carbazole solution in absolute ethanol to 250 ml. with 84% sulfuric acid.

For the determination, layer 1 ml. of sample solution containing not over 0.03 mg. of hexose over 9 ml. of reagent *B*. Similarly prepare an over-all reference blank by layering 9 ml. of reagent *A* with 1 ml. of water. As an unknown blank similarly layer 9 ml. of reagent *A* with 1 ml. of the sample. As a carbazole blank layer 9 ml. of the reagent *B* with 1 ml. of water.

Mix the contents of each of the tubes and heat at 100° for 13 minutes. Cool in ice water for 10 minutes. Thereafter they may stand up to 3 hours before reading. Adjust the spectrophotometer to zero with the over-all reference blank and read the unknown and other blanks against this at 548 mμ for hexoses, 528 mμ for fucose, and 540 mμ for glucuronic acid. Subtract the readings for the unknown blank and the carbazole blank from the reading of the sugar being analyzed. For mixtures of sugars use a corresponding calibration curve.

By thymol.[68] Take a 1-ml. sample containing 0.01-0.1 mg. of sugar in a glass-stoppered tube. Add 7 ml. of sulfuric acid prepared by dilution of 770 ml. of concentrated acid with 230 ml. of water. Let stand for 10 minutes without mixing and cool in an ice bath for 15 minutes. Add 0.1 ml. of 10% thymol solution in absolute ethanol and 0.9 ml. of water. Mix by inversion and heat at 100° for 20 minutes. Immediately place in an ice bath for 5 minutes. Maintain at room temperature for 25 minutes and read at the wave length indicated in Table 14.

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[67] R. E. Glegg, *Anal. Chem.* **28**, 532-4 (1956).

[68] M. R. Sheltar and Y. F. Masters, *Ibid.* **29**, 402-5 (1957).

TABLE 14. WAVE LENGTH OF MAXIMUM ABSORBANCE OF SUGAR-THYMOL-SULFURIC ACID MIXTURES

Sugar	*Max. absorbance,* mμ
Glucose	509
Mannose	513
Galactose	511-12
Fructose	512-13
Sorbose	512-13
Arabinose	493-4
Ribose	494-5
Fucose	503-4
Rhamnose	504
Galaheptose	494-6
Glucoheptose	495-9
Glucuronic acid lactone	491
Furfural	491
Glycogen	509-10
Inulin	513
Turanose	513
Raffinose	512

By picric acid. Blood.[69] Mix a 0.2-ml. blood sample with 1.8 ml. of water and 1 ml. of 1.2% picric acid solution. Filter and mix 1.5 ml. of the filtrate with 0.15 ml. of 10% sodium hydroxide solution. Develop by boiling for 20 seconds or by heating in a 100° bath for 5 minutes.

Urine.[70] If the sample contains more than 1000 ppm. of glucose, deproteinize by adding 6 ml. of 0.2% picric acid solution to a 2-ml. sample. Filter after 10 minutes and develop a 4-ml. aliquot. Otherwise use 1 ml. of the original sample. In either case add 3 ml. of 0.2% picric acid solution and 2 ml. of 10% sodium carbonate solution. Heat for 5 minutes at 100°, cool, and dilute to 25 ml. Read at 530 mμ.

By orcinol.[71] *Glucose and galactose simultaneously.* To a sample containing 0.2 mg. of glucose and galactose, add 0.33 ml. of 1:1 sulfuric acid and dilute with water to 2 ml. Add 2 ml. of chilled orcinol reagent prepared by mixing 4% orcinol solution with an equal volume of 2:3 sulfuric acid. Cool in ice, add 6 ml. of concentrated sulfuric acid, and mix by bubbling nitrogen through. Heat 4 minutes at 67-8°, cool 5 minutes in ice, and read at 470 and 560 mμ.

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[69] Hans J. Wissfeld, *Die Medizinische* **1955**, 712-13.

[70] Gennero Giannettasio, *Laboratorio* 7, 515-7 (1952).

[71] R. G. Hanson and H. W. Norton, *Anal. Chem.* 27, 857-9 (1955).

$$\text{Glucose} = 2.13A_{470} - 1.18A_{560}$$
$$\text{Galactose} = 0.55A_{560} - 0.61A_{470}.$$

Glucose and ribose simultaneously.[72] To prepare the reagent, add 40% ferric chloride solution to concentrated hydrochloric acid to produce a concentration of 0.4% and add orcinol to a concentration of 1%. Mix the reagent with an equal volume of sample containing 0.01-0.1 mg. of glucose and 0.005-0.025 mg. ribose per ml. Heat 40 minutes at 100°, cool, and read at 640 and 680 mμ. Apply simultaneous equations.

By 3,4-dinitrobenzoic acid.[73] As a reagent, dissolve 2 grams of 3,4-dinitrobenzoic acid and 31.8 grams of sodium carbonate in 1 liter of water. Heat an aliquot of glucose solution with 1 ml. of reagent for 10 minutes at 100°. Cool and add 0.2 ml. of concentrated sulfuric acid and 6 ml. of iso-amyl alcohol. Shake to extract the color into the solvent layer. Separate the iso-amyl alcohol layer, and dehydrate with 0.05 gram of anhydrous sodium sulfate. Read at 395 mμ.

By mercuric chloride. Blood.[74] Incubate the deproteinized filtrate from 0.1 ml. of blood for 20 minutes at 45° to remove acetone bodies. Add 4 ml. of 1:49 periodic acid at 25° and mix. After 20 minutes, add 10 ml. of 15% sodium sulfite solution at 25°. After 5 minutes add 2 ml. of 3% mercuric chloride solution. Add 4 ml. of 24% sodium hydroxide solution and mix well. Read at room temperature after 5 minutes.

As molybdenum blue.[75] *Blood.* If the sample is venous blood, mix 5 ml. with a small amount of sodium fluoride. To a 0.2-ml. sample, add 3.4 ml. of water, 0.2 ml. of 10% sodium tungstate solution, and 0.2 ml. of 1:59 sulfuric acid. If deproteinization is incomplete, add 1-2 drops more of sulfuric acid. Centrifuge for 10 minutes.

As cuprotartrate reagent, mix 40 grams of sodium carbonate and 7.5 grams tartaric acid dissolved in 500 ml. of water with 4.5 grams of copper sulfate pentahydrate dissolved in 500 ml. of water. As phosphomolybdate reagent, mix 25 ml. of 30% ammonium molybdate solution, 112.5 ml. of 85% orthophosphoric acid, 75 ml. of 1:3 sulfuric acid, and 37.5 ml. of glacial acetic acid, and dilute to 1 liter with water.

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[72] J. Dubovský, M. Honsová, F. Kalousek, J. Palek, and J. Šonka, *Chem. Listy* **52**, 1796-800 (1958).

[73] T. Takemoto, K. Daigo, and T. Takai, *J. Pharm. Soc. Japan* **75**, 1024 (1955).

[74] M. Takada and Y. Takahashi, *Chiryo* **36**, 604-9 (1954).

[75] Dante Giarnieri, *Rass. med. sper.* **4**, 364-8 (1957).

Mix a 2-ml. sample containing 2-10 mg. of glucose with 2 ml. of the cuprotartrate reagent and 2 ml. of water. Heat at 100° for 10 minutes, cool, and add 2 ml. of phosphomolybdate reagent. Mix, add 12 ml. of water, and read at 490 mμ. To prepare the blank, heat 4 ml. of water with 2 ml. of cuprotartrate reagent at 100° for 10 minutes, cool, and add 2 ml. of phosphomolybdate reagent. Stir, add 12 ml. of water, and read.

By chromotropic acid. Periodate oxidation.[76] To 2 ml. of 6% periodic acid and 2 ml. of 8.4% sodium bicarbonate solution, add 1 ml. of a sample solution equivalent to 0.01-0.1 millimole of formaldehyde after periodate oxidation which is capable of reducing no more than 1 milliequivalent of periodate. Allow the oxidation to proceed for 1 hour at 25°. Add 5 ml. of 6% sodium sulfite solution to reduce excess periodate and dilute to 100 ml. As a blank, add 5 ml. of 6% sodium sulfite solution to a mixture of 2 ml. of 6% periodic acid and 2 ml. of 8.4% sodium bicarbonate solution. Dilute to 100 ml.

To a 1-ml. aliquot of the unknown solution and a 1-ml. blank, add 0.5 ml. of 10% chromotropic acid solution and 5 ml. of 1:6 sulfuric acid. Heat at 100° for 30 minutes in stoppered flasks. Cool and dilute to 50 ml. Bubble through air saturated with water at the rate of 750-1000 ml. per minute to prevent the reduction of color by excess sulfite. Read against a blank at 570 mμ.

By sodium chlorite.[77] *In the presence of fructose.* Choose a sample size depending on the glucose concentration as follows. For samples containing less than 0.5% of glucose in the total solids use a concentration of 100 mg. of sample per ml. for both sample and reference standard. For samples containing 0.5-5% glucose use a concentration of 10 mg. per ml. For samples containing over 5% of glucose, use 0.6 mg. of sample per ml.

Prepare a 6 *M* acetate buffer for pH 4 ± 0.5 by dissolving 590 grams of glacial acetic acid and 302 grams of sodium acetate trihydrate in water and diluting to 1 liter.

Mix the sample and standard with 12.5 ml. of buffer and dilute to about 40 ml. Add exactly 5.00 ml. of 5.4% sodium chlorite solution to each and dilute to 50 ml. Mix, stopper, and place in a constant temperature bath at 25° for 18 hours in the dark. Prepare a glucose-free fructose reference standard the same way. Read at 436 mμ without opening the tubes and with minimum exposure to light.

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[76] J. C. Speck, Jr., and A. A. Forist, *Anal. Chem.* 26, 1942-4 (1954).

[77] F. Stitt, S. Friedlander, H. J. Lewis, and F. E. Young, *Ibid.* 26, 1478-84 (1954).

$$\text{Glucose in mg. per ml.} = 90.1d(B_t - B_c)$$

where

$(B_t - B_c)$ = observed net chlorine dioxide concentration

d = correction factor = 1.063, 1.063, and 1.105 for 0, 10, and 100 mg. per ml. of fructose respectively.

By p-*anisyl tetrazolium blue.*[78] Add to 0.1 ml. of a 1% solution of *p*-anisyl tetrazolium blue, 0.3 ml. of 1.2% sodium hydroxide solution. Add an aliquot of glucose sample containing 0.001 to 0.2 mg. of glucose, and water to a final volume of 1.0 ml. Boil, cool, and add dioxane to a volume of 12.5 ml. for reading.

By 2,3,5-triphenyltetrazolium chloride.[79] To a 4-ml. sample containing about 1 gram of sugar add 1 ml. of 4% sodium hydroxide solution and 1 ml. of 1% solution of the reagent. Incubate at 80° for 6 minutes and cool at once. Add 1 ml. of 1:16.6 acetic acid. Dissolve the red precipitate by careful addition of isopropanol. Dilute to 20 ml. with water, then with 95% ethanol to 100 ml. Read at 490 mμ. The color is stable for 2 hours.

Glucose, fructose, invert sugar, lactose, and maltose as ferric thiocyanate.[80] As a standard dissolve 1.00 gram of analytical-grade iron wire in 10 ml. of 1:10 hydrochloric acid. Add 3 ml. of concentrated nitric acid and dilute to 1 liter. Each ml. corresponds to 0.1 mg. of iron.

Mix 8 ml. of water, 2 ml. of sample solution containing up to 5 mg. of the sugar, 5 ml. of 4% sodium carbonate solution, and 5 ml. of standard ferric chloride solution containing 0.1 mg. of iron per ml. Heat at 100° for 25 minutes and cool rapidly in ice for 45 seconds. Acidify with 25 ml. of concentrated hydrochloric acid, immediately to prevent possible reoxidation of the ferrous iron, and dilute to 100 ml. To a 25-ml. aliquot, add 1 ml. of fresh 25% potassium thiocyanate solution and read after 1.5-2 minutes at 480 mμ.

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[78] N. D. Cheronis and M. C. Zymaris, *Mikrochim. Acta* **6,** 769-83 (1957).

[79] Katsuhiko Amako, *Seito Gijutsu Kenkyukaishi* **7,** 65-9 (1958).

[80] J. K. Roy, *J. Indian. Chem. Soc., Ind. and Eng. News Ed.* **19,** 83-6 (1956).

By enzyme and o-*tolidine. Blood.* To 1.8 ml. of water, add 0.2 ml. of blood, 1 ml. of 1.8% zinc sulfate heptihydrate solution, and 1 ml. of 0.4% sodium hydroxide solution or 1 ml. of 0.85% barium hydroxide solution. Centrifuge briefly at a high speed and transfer 2-ml. and 0.5-ml. portions of the supernatant liquid to tubes of about 18-mm. diameter. The size is to allow for ready diffusion of oxygen into the solution.

To prepare the *o*-tolidine dihydrochloride, add concentrated hydrochloric acid to a solution of the free base in ethanol. Filter the precipitated salt with suction, wash with ethanol and ether, and dry over sodium hydroxide *in vacuo*. Prepare a buffer for pH 4.1 to 4.2 by mixing 1:9 acetic acid and 16% sodium acetate solution to the proper pH. To prepare the composite reagent, add 150 ml. of buffer to 700 mg. of *o*-tolidine dihydrochloride dissolved in a few drops of water. Dissolve 200 mg. of glucose oxidase and 15 mg. of horseradish peroxidase in several ml. of buffer, add to the *o*-tolidine dihydrochloride mixture, and dilute to 300 ml. with buffer. Filter. Reagent blanks made with freshly prepared reagent should have an absorbance of less than 0.01 at 635 mμ.

To the sample solution, add 1.5 ml. of reagent, incubate at 25° for 1 hour, and read at 365 mμ or 635 mμ.

Urine. Mix equal parts by weight of reconditioned air-dried Amberlite IR-120 acid form and Amberlite IR-45 basic form. Slowly add a diluted sample to the resin in a 15-mm. tube held in a slanting position to prevent entrapment of air until the liquid rises above the level of the resin. Refrigerate for 1 hour to prevent bacterial action and separate the urine from the resin. Develop as described for blood from "To prepare the *o*-tolidine. . . ."

By dinitrosalicylic acid. Prepare a reagent containing 1% of dinitrosalicylic acid, 0.2% of phenol, 0.05% of sodium sulfite, and 1% of sodium hydroxide. To a 3-ml. aliquot of the sample containing up to 0.6 mg. of glucose, add 3 ml. of reagent. Heat for 15 minutes at 100° and add 1 ml. of 40% Rochelle salt solution. Cool to room temperature and read at 575 mμ.

Fructose, Levulose

Maxima for direct reading in aqueous solution are at 281.5,[81] 277.5,[82] or 279 mμ.[83] There are numerous aldehydic interferences.

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[81] L. J. Heidt, *J. Franklin Inst.* 234, 473-85 (1942).

[82] L. Kwiecinski and L. Marchlewski, *Bull. intern. acad. polon. sci.* 1927A, 379-94; *Ibid.* 1929A, 317-30.

[83] J. H. Turner, P. A. Rebers, P. L. Barrick, and R. H. Cotton, *Anal. Chem.* 26, 898-901 (1954).

With low temperatures and low acid concentration, it is possible to adapt the anthrone reagent, a common glucose reagent, to the determination of fructose.[84] This method is based on the observation that various sugars show distinctive reaction rates and intensities with anthrone under controlled conditions.[85] The anthrone reagent for this purpose is made up in ethanol to produce a more intense color and prevent the precipitation of anthrone at lower sulfuric acid concentrations. Dilution of the reagent also avoids heat of mixing which causes uncontrolled color formation. Beer's law is followed.

Since the absorbance of the reaction mixture increases at the rate of 0.4% per minute at room temperature, a set of tubes including standards must be read twice in a uniform order, from first to last and then in reverse, taking an average of the two readings for each tube for accuracy. Waiting for an increase in absorbance by fructose causes a greater interference from glucose. The use of a control is required to compensate for the color formation from glucose liberated from dextran by the hydrolytic action of the reagent. In dextran samples, anthrone determines total fructose or nearly so. Much more detail of the application of the anthrone method appears on pages 178 and 194.

The resorcinol method (Vol. III, pp. 207, 211) is modified to allow a greater differentiation between fructose and glucose in mixtures by lowering the reaction temperature to 50° and altering the reaction time to 20 minutes. By this technic 240 parts of glucose are required to give the same absorbance as 1 part of fructose.[86] Ferric chloride is added to hydrochloric acid to intensify the color and minimize the effect of possible traces of iron in the hydrochloric acid.

Preliminary ice-bath cooling eliminates erratic results from variable amounts of reaction before timed periods of heating. Samples containing below 1% of fructose are determined with less accuracy than those containing a higher concentration. In dextran samples only the easily hydrolyzed fructose units are determined by this method. Glucose gives the same color but requires a longer heating period. The absorbance increases as described under anthrone. Therefore a set of tubes is required as described there. A control is also required to compensate for color formed from glucose liberated by the reagent from dextran samples.

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[84] C. S. Wise, R. J. Dimler, H. A. Davis, and C. E. Rist, *Ibid.* **27**, 33-6 (1955).

[85] Leonore Hollander Koehler, *Ibid.* **24**, 1576-9 (1952); T. A. Scott and E. H. Melvin, *Ibid.* **25**, 1656-61 (1953); Cf. R. J. Dimler, W. C. Schaefer, C. S. Wise, and C. E. Rist, *Ibid.* **24**, 1411-4 (1952).

[86] J. H. Roe, *J. Biol. Chem.* **107**, 15-22 (1934); J. S. D. Bacon and D. J. Bell, *Biochem. J.* (London) **42**, 397-405 (1948); D. J. S. Gray, *Analyst* **75**, 314-17 (1950); C. S. Wise, R. J. Dimler, H. A. Davis, and C. E. Rist, *Anal. Chem.* **27**, 33-6 (1955).

So applied, while both anthrone and resorcinol may give part of the fructose in combination in dextran, the anthrone is closer to a total fructose value and the resorcinol reports little combined fructose. This depends on the form of the dextran but even the latter method gives little of that in leucrose.

A pink color is produced by the reaction of guaiacol with hydroxymethylfurfural formed by degradation of fructose by hydrochloric acid.[87] This reaction permits the determination of 0.2-10 mg. of fructose or sucrose in the presence of up to 80 mg. of glucose, other aldohexoses, or aldopentoses.

In concentrated hydrochloric acid a purple color forms from the reaction between fructose and skatole[88] (Vol. III, p. 211). The specificity of this reaction is dependent upon the difference in reaction rates of various carbohydrates at different temperatures. At higher temperatures a greater amount of color is formed by glucose and the reagent relative to that of fructose and the reagent. A low temperature is therefore chosen to lessen the interference of glucose. The major peak of the color is at 510 mμ with a skatole concentration of 0.02-0.04%. A somewhat similar reaction is given by fructose and tryptophane.

Glucose interferes less than with diphenylamine or cysteine-carbazole but the reaction is much less sensitive. The color complex is soluble in chloroform but not in ether. The error encountered when the color intensity increases while the samples are being read in the spectrophotometer is eliminated by reading the optical densities at timed intervals. Beer's law is followed. The reaction is nearly specific for ketoses. Indole reacts similarly.[89]

A violet color is formed by the reaction between fructose and a 0.5% ethanol solution of 3-indoleacetic acid in the presence of hydrochloric acid.[90] The reaction is a condensation of 2 indole rings with chloromethylfurfural or an intermediate reaction product between chloromethylfurfural and the ketose. The furfural derivative is formed by the action of hydrochloric acid on the ketose, and is specific for ketoses, both free and bound in polysaccharides. It is accurate within 1-2% and detects 0.001-0.005 mg. of fructose. The maximum intensity is reached after 15-18 hours and is stable for 30 hours. Heating at 37° accelerates the reaction.

Fructose is determined by sodium nitroprusside in the presence of glucose, mannose, or galactose since only fructose gives a greenish color.[91]

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[87] D. Karácsony, *Élelmezési Ipar* **8**, 309-12 (1954).

[88] Burton M. Pogell, *J. Biol. Chem.* **211**, 143-7 (1954).

[89] M. J. Karvonen and M. Malm, *Scand. J. Clin. and Lab. Invest.* 7, 305-7 (1955).

[90] Antonín Heyrovský, *Chem. Listy* **50**, 1593-7 (1956).

[91] I. G. Bespalov and A. I. Smirnova, *Med. Prom. SSSR* 1949. No. 6, 26-8.

Fructose and sorbose when heated with phosphomolybdic acid and trisodium phosphate form a blue color.[92] The final optical density increases as the time between the addition of the reagent and the addition of the trisodium phosphate increases up to 5 minutes. The color decreases in intensity 2.5-3% over a 21.5 hour period, but the decrease in color is negligible in 1 hour. If the volume of the phosphate reagent is less than that of the reagent or more than 4 times that of the reagent, a green color forms. Other carbohydrates such as starch, raffinose, inulin, maltose, trihalose, cellobiose, lactose, sorbose, galactose, mannose, glucose, xylose, arabinose, and rhamnose gives less color with this reagent. Carbazole-sulfuric acid is also a reagent for fructose.[93]

Fructose is determined in the presence of aldose sugars by either *p*-ansidine or 3,3′-dimethoxybenzidine in phosphoric acid.[94] The 3,3′-dimethoxybenzidine reagent produces a slight color in the phosphoric acid which fades upon standing and does not interfere, and this reagent also produces a more colored blank than with *p*-anisidine. However, except for these considerations, both reagents give good results with fructose. Since the reagent hydrolyzes higher saccharides it is possible to determine fructose in disaccharides and polysaccharides without previous hydrolysis. A correction must be applied for the presence of sucrose. Glucose does not interfere. Mannose, arabinose, xylose, and rhamnose develop slight colors with the reagents while sorbose, tagatose, sedoheptulose, and lactulose interfere. Up to 25 mg. of sodium chloride, phosphate, and acetate can be tolerated. Fructose is extracted from plant samples with alcohol. Beer's law is followed for 0.05-0.3 mg. fructose.

Fructose is determined in many cases by a reagent used for other sugars. As a specific reagent so used see determination by chromotropic acid, page 196. For determination by consumption of periodic acid see page 174. For determination by orcin see page 209. For determination by thymol see page 197. For determination in the presence of xylulose by resorcinol and ferric ion see page 172. For determination by sodium chlorite see page 200. For determination by phenol in sulfuric acid see page 167. For determination by reduction of ferric ion see page 201. For determination in 90% sulfuric acid see page 168 or in 87% sulfuric acid see page 161. For determination by carbazole in sulfuric acid see page 168. For determination by *N*-ethylcarbazole see page 168. For determination by 2,3,5-triphenyltetrazolium chloride see page 201. For determination by 3.5-dinitrosalicylic acid see page 202.

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[92] F. J. T. Harris, *Analyst* 78, 287-90 (1953).

[93] L. Ujejski and E. R. Waygood, *Can. J. Chem.* 32, 14-8 (1954).

[94] Lyle E. Hessler, *Anal. Chem.* 31, 1234-6 (1959).

Samples—*Dextran.*[95] Dissolve a sample weighing up to 60 mg. containing 0.025-0.1 mg. of fructose in 2 ml. of water, or dilute such a liquid sample to 2 ml. If the fructose content is below 10% of the sample use a control containing the same type of dextran in the same concentration as the sample. It is not necessary to filter hazy solutions. Develop with alcoholic anthrone.

Alternatively, dissolve a sample up to 500 mg. containing 0.2-0.8 mg. of fructose in 2 ml. of water, or dilute such a liquid sample to 2 ml. Develop with resorcinol.

Procedure—*By alcoholic anthrone.* As a reagent, slowly add with cooling 100 ml. of concentrated sulfuric acid to 60 ml. of absolute ethanol. At room temperature add 200 mg. of anthrone. The reagent is stable under refrigeration for 6 weeks.

Cool the sample solution containing 0.025-0.1 mg. of fructose in ice and layer in 8 ml. of cold reagent. Stir well and heat at 50° for 20 minutes. Cool in ice for 1 minute and read at 620 mμ against a reagent blank. Include a 0.06-mg. standard with the determination and read the unknown, control, and standard in order, then in reverse and use an average of the 2 readings. Calculate the fructose as follows:

$$K = \frac{\text{mg. of fructose in the standard}}{\text{absorbance of the standard}}$$

$$\text{weight of fructose} = K\,(\text{absorbance of the unknown} - \text{absorbance of the control}).$$

By resorcinol. To a cooled 2-ml. aliquot containing 0.2-0.8 mg. of fructose, add 5 ml. of cold concentrated hydrochloric acid containing 0.0124 gram of ferric chloride hexahydrate per liter to intensify the color and minimize the effect of possible traces of iron in the acid. Add 2 ml. of cold 0.1% resorcinol solution in absolute ethanol, mix, and heat at 50° for 20 minutes. Cool in ice for 1 minute and read at 505 mμ against a reagent blank.

Run a fructose standard and a control containing the same type and concentration of dextrans as the sample together with the unknown. If the solutions are hazy prepare a haze blank by replacing the reagent with cold absolute ethanol. Calculate the fructose as follows:

$$K = \frac{\text{mg. of fructose in the standard}}{\text{absorbance of the standard}}$$

$$\text{weight of fructose} = K\,[A_{\text{unknown}} - A_{\text{unknown haze blank}} - A_{\text{control}}].$$

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[95] C. S. Wise, R. J. Dimler, H. A. Davis, and C. E. Rist, *Ibid.* 27, 33-6 (1955).

By guaiacol. Reflux a 2-ml. aliquot containing 0.2-10 mg. of fructose or the equivalent as sucrose, 2 ml. of 20% guaiacol solution in absolute ethanol, and 10 ml. of 2:3 hydrochloric acid in 95% ethanol for exactly 10 minutes. Cool in cold water for 5 minutes and read the pink color at once.

By skatole. Place a 1-ml. sample containing up to 0.27 mg. of fructose, a reagent blank, and a fructose standard of about 0.15 mg. in an ice bath. To each add 1 ml. of 0.04% skatole solution in 95% ethanol and 8 ml. of 5:1 hydrochloric acid. Mix and place individually at 30-second intervals in a water bath at 37° for 40 minutes. Read each against a water blank at 510 mμ at 30-second intervals.

By indole. To 0.5 ml. of protein-free sample containing at least 0.005 mg. of fructose, add 0.5 ml. of a reagent containing 25 mg. of indole per 100 ml. of 0.2% aqueous benzoic acid. Add 5 ml. of concentrated hydrochloric acid and heat at 50° for 20 minutes. Cool and read at 470 mμ.

By sodium nitroprusside. To a 3-cu. mm. sample containing 2-10% of fructose, at pH 3.4, add 1 ml. of fresh 1% sodium nitroprusside solution and 10 drops of 4% sodium hydroxide. After 15 minutes, add 5 drops of glacial acetic acid. Mix, allow 20 minutes for full color development, and read.

By phosphomolybdic acid. As a reagent boil a mixture of 100 grams of sodium tungstate, 20 grams of phosphomolybdic acid, 100 grams of 85% phosphoric acid, and 700 ml. of water for 2 hours. Cool, filter if necessary, and dilute to 1 liter.

Mix a 10-ml. sample containing fructose and/or sorbose with 5 ml. of reagent. After 5 minutes, add 10 ml. of 10% trisodium phosphate solution, and heat at 100° for 10 minutes. Dilute immediately with cold water to 95 ml., cool, and dilute to 100 ml. Read at wave lengths up to 675 mμ against a reagent blank.

By carbazole-sulfuric acid. To a 1-ml. sample containing up to 0.02 mg. of fructose, add 0.2 ml. of a 1.5% solution of cysteine in concentrated hydrochloric acid, and 6 ml. of 2:3 sulfuric acid. Immediately add 0.2 ml. of 0.12% carbazole solution in absolute ethanol. After standing for 1-2 hours read at 560 mμ.

By p-*ansidine.* Dilute a sample containing 0.05-0.3 mg. of fructose to 2 ml. and add 4 ml. of 0.5% *p*-ansidine solution in 85% phosphoric acid.

Heat to boiling, cool to room temperature, let stand for 30 minutes, and read at 450 mμ against a reagent blank.

2-DESOXYGLUCOSE

A quinaldine reagent reacts with α-methylene aldehydes.[96] This is applicable to determination of 2-desoxyglucose.[97] There is no color with 2-desoxypentoses, probably because of their extreme acid sensitivity. Therefore desoxyribonucleic acid and its decomposition products do not interfere. Up to 5 mg. of glucose and natural ingredients of deproteinized blood do not interfere. Dilute urine 1:10 before analysis. The results with rat urine contaminated with food and feces are not reliable.

Procedure—Mix 4 ml. of 0.45% zinc sulfate solution and 0.8 ml. of 0.4% sodium hydroxide solution, adjusted as is usual for Somagyi's reagent to be equivalent. Add 0.1 ml. of blood, urine, or other sample containing 0.01-0.15 mg. of 2-desoxyglucose. Heat at 100° for 3 minutes and cool in water. Filter through a dry paper.

As reagent dissolve 1 gram of 3,5-diaminobenzoic acid dihydrochloride in 25 ml. of 85% phosphoric acid and 60 ml. of water. Filter with charcoal if colored. Mix 3 ml. of deproteinized sample with 3 ml. of reagent. Heat at 100° for 15 minutes and cool for 15 minutes in cold water. Read at 420 mμ against a reagent blank.

GALACTOSE

In the presence of glucose it is removed by glucose oxidase and the galactose developed in buffered solution with a copper complex and phosphomolybdic acid.[98] The most satisfactory method of deproteinizing is by barium hydroxide and zinc sulfate. Anthrone is an alternative reagent for development.[99]

When glucose, galactose, lactose, xylose, and arabinose are separated on a paper chromatogram they are developed on the paper with 9.3% diphenyl amine—9.6% citric acid in 50% ethanol. Thereafter the eluate of the spots is read at 420 mμ.[100]

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[96] L. Valluz, G. Amiard, and M. Pesez, *Bull. soc. chim. France* **1948**, 680-1.

[97] F. B. Cramer and G. A. Neville, *J. Franklin Inst.* **256**, 379-81 (1953).

[98] N. Tygstrup, K. Winkler, E. Lund, and H. C. Engell, *Scand. J. Clin. and Lab. Invest.* **6**, 43-8 (1954).

[99] G. Sondergaard, *Ibid.* **10**, 203-10 (1958).

[100] J. W. Date, *Ibid.* **10**, 149-54 (1958).

For the simultaneous determination of galactose and glucose by orcinol, see page 198. For determination by phenol in sulfuric acid see page 167. For determination by aminodiphenyl see page 168. For determination by anthrone see page 194. For determination by orcinol see page 170. For determination by consumption of periodic acid see page 174. For determination by chromotropic acid see page 196. For determination by thymol see page 197. For determination in 90% sulfuric acid see page 179. For determination by the color in 79% sulfuric acid see page 168. For determination by carbazole in sulfuric acid see page 197. For determination by *N*-ethylcarbazole see page 197. For determination by 3,4-dinitrosalicylic acid see page 202.

Procedure—*Plasma in the presence of glucose.* Shake 2 ml. of plasma with 0.5 mg. of notatin (corylophyline) to oxidize the glucose. Add 2 ml. of 2.72% monopotassium phosphate solution as buffer. Precipitate proteins[101] with 0.2 ml. of 21% ferric sulfate solution neutralized with barium carbonate. Filter and treat 2 ml. of the filtrate with a 2.4% solution of copper acetate in 0.2% lactic acid. Heat in boiling water for 8 minutes and cool.

As phosphomolybdic acid reagent boil 150 grams of molybdenum trioxide and 75 grams of sodium carbonate with 500 ml. of water. Filter insoluble matter, add 300 ml. of 85% phosphoric acid, and dilute to 1 liter. Add 2 ml. of this reagent to the heated and cooled sample. Read after 2 minutes.

SEDOHEPTULOSE AND SEDOHEPTULOSAN

Orcin (Cf. Vol. III, pp. 213-4) in trichloroacetic acid forms a blue-green color with sedoheptulose and a yellow color with fructose and galactose.[102] Sugars in blood, serum, erythrocytes, and tissue homogenates can be determined with this reagent, but it is unsuitable for urine. Other sugars do not react.

Carbazole-sulfuric acid produces a red-brown color with sedoheptulosan with a maximum absorption at 490 mμ after heating.[103] Beer's law is followed for 0.01-0.03 mg. per ml. Sedoheptulosan can be determined by this method in the presence of ribose and fructose.

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[101] A. Steiner, F. Urban, and E. S. West, *J. Biol. Chem.* **98**, 289-93 (1932).

[102] J. Šonka and M. Kuchařová, *Chem. Listy* **50**, 388-90 (1956).

[103] L. Ujejski and E. R. Waygood, *Can. J. Chem.* **32**, 14-8 (1954).

Procedure—*Sedoheptulose by orcin.* To prepare the reagent, dissolve 80 grams of trichloroacetic acid in water, add 0.5 gram of orcin, and dilute to 100 ml. Deproteinize the sample with an equal volume of 20% trichloroacetic acid, centrifuge, and treat a 2-ml aliquot of the supernatant liquid with 3 ml. of reagent. Heat at 100° for 1 hour, cool, and read sedoheptulose at 620 mμ.

Sedoheptulosan by carbazole-sulfuric acid. Ribose and fructose absent. As reagent reflux 8.35 ml. of 0.5% carbazole solution in absolute ethanol and 241.65 ml. of 0.002% potassium persulfate solution with concentrated sulfuric acid and dilute to 85% of sulfuric acid by weight.

To 1 ml. of sample containing 0.01-0.03 mg. of sedoheptulosan add 9 ml. of the carbazole-sulfuric acid reagent. Heat for 13 minutes at 100° and cool for 10 minutes in ice water. Read at 490 mμ.

Ribose and fructose present. To 0.9 ml. of sample containing up to 0.02 mg. of ribose or fructose per ml. add 0.1 ml. of 3% solution of cysteine in concentrated hydrochloric acid. Add 5 ml. of 2:3 sulfuric acid and 0.2 ml. of 0.12% solution of carbazole in 95% ethanol. Heat for 10 minutes at 100°, cool, and let stand for 24-48 hours. Read at 510 mμ.

CHAPTER 9

POLYSACCHARIDES

The polysaccharides vary in complexity upward from sucrose, a disaccharide, through varying degrees of complexity to cellulose. The methods of determination of hexoses and pentoses are applicable to many of them by hydrolysis to the monosaccharides. Water-soluble fractions of this group as a whole are estimated with anthrone, page 194. For determination of disaccharides by iodine see page 183.

For determination of trihalose by the color in 79% sulfuric acid see page 168. For determination by *N*-ethylcarbazole see page 168. For inulin in chicory roots, extract and determine by the fructose content, reading it as molybdenum blue,[1] page 207. Cellobiose, the disaccharide from hydrolysis of cellulose, can be determined by aminodiphenyl, page 168. For determination by consumption of periodic acid see page 174.

For determination of melibiose by aminodiphenyl see page 168. For determination by the color in 79% sulfuric acid see page 168. For determination by *N*-ethylcarbazole see page 168.

For determination of turanose by thymol see page 197. For determination by anthrone see page 194. For determination by *N*-ethylcarbazole see page 168.

For determination of melezitose by anthrone in the presence of some other sugars see page 194. For determination by aminodiphenyl see page 168. For determination by the color in 79% sulfuric acid see page 168. For determination by *N*-ethylcarbazole see page 168.

For determination of raffinose by sodium chlorite see page 200. For determination by anthrone see page 194. For determination by phenol in sulfuric acid see page 167. For determination by aminodiphenyl see page 168. For determination by thymol see page 197. For determination by the color in 79% sulfuric acid see page 168.

Yeast mannan, a polysaccharide from baker's yeast autolyzate, is determined with concanavalin-*A* in the range of 0.01-0.1 mg. For discussion and procedure, see glycogen, page 218. For determination of maltose by 3,4-dinitrosalicylic acid see page 202.

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[1] J. Trzebinski and W. Gmika, *Roczniki Nauk Rolniczych* Ser. A. **74**, 629-40 (1957)

SUCROSE

Resorcinol in concentrated hydrochloric acid forms a dark red color with sucrose (Vol. III, p. 216) which permits the determination of 0.1-1 mg. of sucrose.[2] For sirups containing sucrose or sucrose and glucose, the sucrose is first hydrolyzed in hydrochloric acid. The glucose is then oxidized by oxygen in the presence of glucose oxidase to produce gluconic acid and hydrogen peroxide. The hydrogen peroxide reacts with an organic substrate in the presence of horseradish peroxidase to produce a colored product, thus giving a specific method for glucose, and indirectly for sucrose.[3]

The oxygen content of the enzyme regulates the rate of color formation and differing surface areas of the enzyme in the tubes would cause unequal atmospheric oxygen diffusion. Since hydrochloric acid is used to stop the enzyme reaction, its addition must be at an exactly timed interval. If the sample is of sucrose sirup dilute to an equivalent of about 4 mg. of glucose per ml. If glucose is to be determined in the presence of sucrose dilute to contain about 0.2 mg. per ml. before hydrolysis.

For determination of sucrose by anthrone (Vol. III, p. 215) see page 194. For determination by aminodiphenyl see page 168. For determination by consumption of periodic acid see page 174. For determination by chromotropic acid see page 200. For determination by sodium chlorite see page 200. For determination by guaiacol see page 207. For determination in 90% sulfuric acid see page 179. For determination by the color in 79% sulfuric acid see page 168. For determination by *N*-ethylcarbazole see page 168.

Procedure—*By resorcinol. Milk.* Dilute a 50-ml. sample to 100 ml. with 1:2 hydrochloric acid, shake, and filter. To a 5-10 ml. aliquot containing 0.1-1 mg. of sucrose, add 5 ml. of resorcinol reagent prepared by diluting 0.5 gram of resorcinol dissolved in 100 ml. of concentrated hydrochloric acid to 300 ml. with water. Heat at 100° for 15 minutes and dissolve any precipitate with 95% ethanol. Read at 480 mμ. The color is stable for several days.

By peroxidase. Glucose plus sucrose. Dilute to the equivalent of approximately 4 mg. of glucose per ml. Heat a 5-ml. sample, 25 ml. of water, and 5 ml. of concentrated hydrochloric acid at 100° for 30 minutes. Cool, adjust the pH to 5.6, and dilute to 100 ml. with water.

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[2] Renato Bagnulo, *Latte* **28**, 82 (1954).

[3] J. P. Comer and H. F. Brickley, *Anal. Chem.* **31**, 109-10 (1959).

As a reagent, dissolve 2900 units of glucose oxidase, 1815 Pz units of horseradish peroxidase, 0.125 gram of dianisidine citrate (prepared by addition of an ether solution of citric acid to an ether solution of dianisidine), 0.2 gram of citric acid monohydrate, and 1.2 grams of sodium citrate dihydrate in 500 ml. of water. Filter before using.

Prepare a 1-ml. water blank and three 1-ml. standard glucose blanks containing 0.2 mg. of anhydrous dextrose per ml. The latter must have been left standing for 1 hour to establish equilibrium between α- and β-glucose. Take a 1-ml. aliquot of the sample. If the sample is highly colored, prepare another blank containing 1 ml. of 1:1 hydrochloric acid. Add 10 ml. of enzyme reagent to each tube at timed intervals and add 1 ml. of 1:1 hydrochloric acid to each tube exactly 10 minutes later, except for the blank tube containing the hydrochloric acid, to which is added 1-ml. aliquot of the sample at that time. Mix by agitation and read at 400 mμ against the blank containing water and reagent. Calculate as total sucrose and, if desired, correct for the glucose content as determined below.

Glucose in the presence of sucrose. Dilute the original sample to contain about 0.2 mg. of glucose per ml. Develop as above without the hydrolysis step.

Lactose

Phenol in the presence of sulfuric acid as a reagent for the determination of lactose has been applied to milk and cheese samples.[4] The general reaction is applicable to many forms of sugar samples, page 162. Casein interferes but lactic acid, citric acid, and butter fat do not. Beer's law is followed, and the color is stable for 1 hour.

Another method for lactose in milk is based on the reduction of ferric chloride in sodium carbonate solution.[5] The per cent of lactose in the sample is equal to the amount of ferric ion reduced multiplied by a factor of 1.22.

The reducing power of lactose is the basis of its determination with the hexavalent chromium ion. When heated in an alkaline solution with potassium chromate, lactose produces a green color due to reduced chromium, the color intensity rising until the alkalinity of the solution reaches

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[4] A. J. G. Barnett and G. A. Tawab, *J. Sci. Food Agr.* **8**, 437-41 (1957); D. R. Erickson and G. A. Richardson, *Proc. 38th Ann. Meeting Western Div. Am. Dairy Sci. Assoc.* **1957,** 6 pp.

[5] S. N. Mitra and J. K. Roy, *J. Indian Chem. Soc., Ind. & News Ed.* **18,** 34-8 (1955).

0.4 *N*. Beer's law is followed. Other reducing sugars interfere.[6] When applied to mixtures of lactose and saccharose the latter does not react.[7] By acid hydrolysis the saccharose can then be determined by difference.

Lactose can be used to reduce ferricyanide ion to ferrocyanide and the latter converted to the blue ferric compound for reading in suspension.[8] When glucose, galactose, lactose, xylose, and arabinose are separated on a paper chromatogram they are developed on the paper with 9.3% diphenyl amine—9.6% citric acid in 50% ethanol. Thereafter the eluate of the spots is read at 420 mμ.[9]

For lactose by chromotropic acid see page 196. For determination by aminodiphenyl see page 168. For determination by consumption of periodic acid see page 174. For determination by the color in 79% sulfuric acid see page 168. For determination by *N*-ethylcarbazole see page 168. For determination by reduction of ferric ion see page 201. For determination of lactose by 3,4-dinitrosalicylic acid see page 202. The anthrone reaction is also applicable by rapid addition of the reagent.[10]

Samples—*Milk*. Dilute a 1-gram sample to 1 liter with water and filter. Determine with phenol in sulfuric acid. Alternatively, warm a 10-gram sample in 200 ml. of water and add 1 ml. of 1:9 acetic acid. Heat for 30 minutes, cool, dilute to 250 ml., and filter. Develop by reduction of ferric chloride. For development with chromate, separate interfering substances from the sample by adding an aqueous solution containing 1% of aluminum sulfate and 0.9% of sodium hydroxide saturated with sodium fluoride as a protein coagulant. Let stand for 15 minutes, filter, and develop.

Cheese. Extract a ground 1-gram sample with six successive 10-ml. portions of water. Dilute the extracts to 1 liter. Filter and develop with phenol and sulfuric acid.

Procedure—*By phenol and sulfuric acid.*[11] Dilute an aliquot containing up to 0.1 mg. of lactose to 2 ml. Add 6 drops of a phenol reagent prepared by dissolving 80 grams of phenol in 20 ml. of water. Add 5 ml. of sulfuric acid and read at 490 mμ against a reagent blank.

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[6] M. N. Tul'chinskiĭ, *J. Appl. Chem. USSR* 23, 181-5 (1950); 24, 178-84 (1951)

[7] M. N. Tul'chinskiĭ, *Zhur. Anal. Khim.* 11, 742-5 (1956).

[8] J. C. Godfrain, P. Bertrand, and L. Liandier, *Lait* 39, 32-6 (1959).

[9] J. W. Date, *Scand. J. Clin. and Lab. Invest.* 10, 149-54 (1958).

[10] Tomoya Nakata, *Tokyo Jikeikai Ika Daigaku Zasshi* 72, 464-70 (1957).

[11] A. J. G. Barnett and G. A. Tawab, *J. Sci. Food Agr.* 8, 437-41 (1957).

By reduction of ferric chloride.[12] To a 5-ml. aliquot, add 5 ml. of 0.05% ferric chloride hexahydrate solution and 3 ml. of 0.02% sodium carbonate solution. Add 7 ml. of water and heat at 100° for 25 minutes. Determine unreduced ferric ion by the thiocyanate method (Vol. II, p. 307 *et seq.*, Vol. IIA, pp. 229-31). Deduct the mg. of unreduced ferric iron from the total added, and multiply the reduced iron by a factor of 1.22 to determine lactose.

By chromate. Mix a 3-ml. sample with 6 ml. of 1.6185% potassium chromate solution and 3 ml. of 10.6% sodium carbonate solution. Heat at 100° for 15 minutes and read the green trivalent chromium at 420 or 590 mμ.

INULIN

The hydrolysis of inulin yields fructose which reduces phosphomolybdic acid to molybdenum blue.[13] Beer's law is followed at 0.01-0.04 mg. of inulin per ml. The interference of glucose is minimized by the fact that its reducing power in this method is approximately one-thirtieth of that of fructose.

For determination of inulin by thymol see page 197. For determination by the color in 79% sulfuric acid see page 186. The reaction with anthrone and glucose (page 194) can be applied in blood and urine by heating for 10 minutes at 55° and a further 10 minutes at 100°, with corrections.[14] The maximum absorbancy is at 625 mμ.

Procedure—*Serum.* Mix a 2-ml. sample with 10 ml. of water, 6 ml. of 3.5% cadmium sulfate octahydrate solution, and 2 ml. of 4% sodium hydroxide solution. Heat briefly to 100° and filter after 10-15 minutes.

To prepare the reagent follow the procedure under glucose as molybdenum blue, Vol. III, p. 205. To a 2-ml. aliquot containing 1-4 mg. of inulin add 2 ml. of reagent and heat for 30 minutes at 100°. Cool for 5 minutes and add 6 ml. of 1:35 sulfuric acid. Mix well and read at 700 mμ.

Urine. Take a sample diluted 1:49 and follow the procedure under serum.

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[12] S. N. Mitra and J. K. Roy, *J. Indian Chem. Soc., Ind. & News Ed.* **18**, 34-8 (1955).

[13] H. Frank and F. Scheiffarth, *Klin. Wochschr.* **34**, 914-8 (1956).

[14] J. Führ, J. Kaczmarczyk, and C. D. Krüttgen, *Ibid.* **33**, 729-30 (1955); Cf. Tomoya Nokata, *Tokyo Jikeikai Ika Daigaku Zasshi* **72**, 460-3 (1957).

DEXTRAN

Since dextran, a neutral polyglucide, yields glucose units on hydrolysis, anthrone, a common glucose reagent, can be adapted to its determination.[15] In blood and urine samples, dextran is precipitated from a trichloroacetic acid solution by alcohol prior to determination by anthrone. Trichloroacetic acid performs many functions: acting as a protein precipitant, providing an acid solution of suitable ionic concentration to permit precipitation of dextran by alcohol, and preventing the precipitation of phosphates which sorb or contain anthrone-sensitive materials.[16]

A standard must be run with the sample since the age of the anthrone reagent has an effect on the color intensity formed. The inclusion of a standard also eliminates error from variation in concentration of acid in the reagent, heating time, and bath temperature. The reagent may be mixed with the sample in a cold water bath to minimize the effect of rise in temperature. The color is stable in the dark for 2 hours with no decrease in intensity. When exposed to direct sunlight, the color fades 14% within 1 hour.

Addition of 0.2 ml. of 10% alkyl dimethylbenzylammonium chloride solution or 2 mg. of phenyl mercuric acetate to each 100 ml. of standard solution act as preservatives against deterioration from microbial contamination. Substances which react with anthrone, such as fructose, interfere. If the concentration of sodium chloride is twice that of dextran the results are high due to the chloride ion. Sodium sulfate does not interfere. The presence of 0.001-0.002 mg. of extraneous carbohydrate material will cause a 1% error. Cellulose fiber also interferes. Beer's law is followed.

Dextran which has been precipitated with alcohol from a trichloroacetic acid solution can be read turbidimetrically at 440 [17] or 720 [18] mμ. Dextran is also determined by phenol in sulfuric acid, page 167.

Samples—*Blood plasma.* Deproteinize the sample with 5% trichloroacetic acid solution. Dilute, so that the final solution contains 0.01-0.2 mg. of dextran per ml., and filter. Precipitate dextran by adding 5 ml. of 95% ethanol to 1 ml. of the filtrate. Cover and heat at 37-40° for 3 hours or leave at room temperature overnight. Centrifuge for 15 minutes. Decant, invert the tubes for 15 minutes to drain, and add 2 ml. of water down the sides to wash the tubes. Tap to dislodge the dextran mat and disperse the dextran. Develop with anthrone.

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[15] T. A. Scott, Jr., and E. H. Melvin, *Anal. Chem.* **25**, 1656-61 (1953).

[16] Joseph H. Roe, *J. Biol. Chem.* **208**, 889-96 (1954).

[17] L. Jacobsson and H. Hansen, *Scand. J. Clin. and Lab. Invest.* **4**, 352-4 (1952).

[18] W. Metcalf and L. B. Rousselot, *J. Lab. Clin. Med.* **40**, 901-6 (1952).

Urine. Dilute a sample with 5% trichloroacetic acid solution and filter if protein is present. Follow the procedure as for blood plasma starting with "Dilute, so that the final solution. . . ."

Procedure—*By anthrone.*[19] To a 2-ml. sample containing up to 0.2 mg. of dextran, add 10 ml. of 0.05% anthrone reagent in 3:2 sulfuric acid. Mix thoroughly and close with a stopper containing a small glass vent-tube. Heat for 13 minutes at 100° and cool in water to room temperature. Read at 620 mμ against a reagent blank.

Calculate dextran as mg. per 100 ml. by $DU/DS \times 0.1 \times$ dilution of plasma $\times 100 \times 0.9$

where

DU = optical density of the unknown
DS = optical density of the standard
0.1 = mg. of glucose in 2 ml. of standard solution
0.9 = factor for converting glucose value to dextran value.

Alternatively,[20] add 10 ml. of 0.2% anthrone reagent solution in concentrated sulfuric acid to a test tube maintained at 10-15°. Add a 5-ml. sample containing 2-4 mg. of dextran per 100 ml. to form a layer above the reagent. Shake vigorously, keeping immersed in a cold water bath, and let stand to reach room temperature. Heat at 90° for 16 minutes and cool to room temperature under water. Read immediately against a water blank similarly treated at 625 mμ. Run a standard blank with the sample and calculate the dextran as follows:

$$C_x = \frac{C_{\text{std}}\,(D_x - D_b)}{D_{\text{std}} - D_b}$$

where

C_x = concentration of dextran sample multiplied by the appropriate dilution factor
C_{std} = concentration of dextran standard
D_x = absorbance of sample
D_b = absorbance of blank
D_{std} = absorbance of standard.

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[19] Joseph H. Roe, *J. Biol. Chem.* **208**, 889-96 (1954).

[20] T. A. Scott, Jr., and E. H. Melvin, *Anal. Chem.* **25**, 1656-61 (1953).

Turbidimetrically. To 1 ml. of deproteinized sample containing 0.01-0.2 mg. of dextran add 10 ml. of 95% ethanol. Let this stand for 1 hour at room temperature and read at 720 mμ against water. Alternatively use 1 ml. of sample and 10 ml. of 95% ethanol and read at once at 440 mμ.

GLYCOGEN

The common carbohydrate reagent, anthrone, is applied to the analysis of glycogen.[21]

The interaction of concanavalin-*A*, a globulin extracted from jack bean meal, with glycogen is the basis for a turbidimetric method.[22] A range of 0.1-1 mg. of glycogen is chosen since there will be no rapid precipitation of glycogen in this range. As an added precaution polyvinyl alcohol is added as a stabilizer. Higher concentrations of glycogen will precipitate, however, even in the presence of the stabilizer. Control of pH between 5 and 6 is important since higher or lower pH produces little or no turbidity.

Yeast mannan is determined in the presence of glycogen by the addition of diluted saliva to the mixture of the polysaccharides, the glycogen being destroyed by the salivary α-amylase. Similarly, it is possible to determine glycogen in mixtures of the two polysaccharides by determining the total mixture with concanavalin-*A*, following with an addition of salivary α-amylase, determination of the yeast mannan, and subtraction to estimate the glycogen. Proteins do not interfere and the polysaccharide therefore does not have to be separated. Oxidizing agents interfere. The reagent is specific.

When liver is homogenized with trichloroacetic acid and physiological saline, the filtrate can be read nephelometrically. For the purpose add 1.5 ml. of 95% ethanol to 1.2 ml. of sample solution and read after 3 hours.[23] For determination of glycogen by thymol see page 197. For determination by the color in 79% sulfuric acid see page 168. It is also determined by the color in 90% sulfuric acid.[24]

Samples—*Mouse genital organs.*[25] Heat a sample of up to 200 mg. less than 2 minutes after the death of the animal in 0.4 ml. of 30% potassium hydroxide solution for 30 minutes at 100°. Precipitate glycogen with 0.8 ml. of 95% ethanol. Heat to boiling, cool 5-10 minutes in a refrigerator, and centrifuge for 30 minutes. Decant and drain for 15 minutes. Extract glycogen from the precipitate with 1 ml. of 5% trichloroacetic acid solution and centrifuge the extract for 20 minutes. Repeat the extraction with 1.5 ml. of 5% trichloroacetic acid solution and develop the combined extracts with anthrone. Prepare a blank using tissue incubated for 1 hour in phosphate Ringer solution at pH 7.4 to destroy glycogen.

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[21] R. Peterson and D. Rose, *Can. J. Technol.* 29, 317-21 (1951); John Kahan, *Arch. Biochem. and Biophys.* 47, 408-18 (1953); H. W. Payne and J. P. A. Latour, *J. Clin. Endocrinol. & Metabolism* 15, 1106-15 (1955); J. H. Balmain, J. D. Biggers and P. J. Claringbold, *Australian J. Biol. Sci.* 9, 139-46 (1956).

[22] J. A. Cifonelli and F. Smith, *Anal. Chem.* 27, 1639-41 (1955).

[23] L. Gyermek and G. Fekete, *Nature* 175, 386 (1955).

[24] R. Suzulsi and N. Arakawa, *Nippon Nôgei Kagaku Kaishi* 32, 631-5 (1958).

[25] J. Balmain, J. D. Biggers, and P. J. Claringbold, *Australian J. Biol. Sci.* 9, 139-46 (1956).

Endometrial tissue.[26] Digest in 30% potassium hydroxide solution by heating for 30 minutes at 100°. Dilute an aliquot of the digest and adjust the pH to 4. Treat with 2 volumes of 95% ethanol and refrigerate for 45 minutes. Develop with anthrone.

Procedure—*By anthrone.* To a sample containing at least 0.005 mg. of glycogen, add 5 ml. of 0.2% anthrone solution in concentrated sulfuric acid with cooling. Heat for 10 minutes at 100° and read at 620 mμ.

By concanavalin-A.[27] To prepare the reagent, stir 20 grams of jackbean meal with 200 ml. of 2% sodium chloride solution for 10 minutes, followed with centrifuging for 10 minutes. Treat the turbid supernatant liquid with 2 ml. of 14.4% ammonium acetate solution at pH 4.2. Let stand for 1 hour and centrifuge. Heat to 50-55° with continual stirring, let stand at room temperature for 30 minutes, and centrifuge. If the solution is not clear, add 2-3 ml. of 0.1% glycogen solution, let stand in the refrigerator overnight, and filter or centrifuge. Treat the clarified solution with 15 ml. of 7% polyvinyl alcohol solution and refrigerate until used. The reagent which is stable for several months when refrigerated, should be clarified before use.

To a 1-ml. sample containing 0.1-1 mg. of glycogen add 9 ml. of reagent and let stand for 10 minutes at room temperature. Read at 420 mμ against a water blank. The result is glycogen and yeast mannan. To obtain yeast mannan alone, destroy the glycogen with saliva.

STARCH

In the analysis of apples, the starch fraction which is actually determined is the amylose fraction. In this case the success of the iodine method (Vol. III, pp. 231-2) depends on the constancy of the ratio of amylose to amylopectin starch since the iodine forms 6 times as much color with amylose as with amylopectin.[28] Perchloric acid is commonly used as a starch solvent since it extracts starch without hydrolysis to sugar. An alternative is nearly saturated calcium chloride solution faintly acidified with acetic acid.[29]

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[26] H. W. Payne and J. P. A. Latour, *J. Clin. Endocrinol. and Metabolism* **15**, 1106-15 (1955).

[27] J. A. Cifonelli and F. Smith, *Anal. Chem.* **27**, 1639-41 (1955).

As in the determination of other carbohydrates, anthrone is a reagent for starch.[30] Use of a diluted reagent lessens the rise in temperature upon the addition of the reagent to an aqueous solution, thus reducing some error. To compensate for possible variations in different batches of the anthrone reagent and for possible presence of interfering agents in extracts of the test material, an internal glucose standard is introduced. Fructose, glucose, dextrin, and certain pentoses react with the reagent. α-Cellulose, protein, and mineral materials do not interfere. Up to 0.3 mg. of starch conforms to Beer's law

Starches in concentrations less than 250 ppm. are determined indirectly by the dichromate remaining following an oxidation of the starch.[31] The consumption of dichromate per unit weight of the various starch produces is independent of the complexity of the starch whereas each type of starch gives slightly different results with permanganate.

The rate and extent of oxidation are functions of dichromate and acid concentrations, temperature, and time. The acid concentration should be below 4% and the dichromate concentration in the final reaction mixture at least 0.002 M. The reaction time and temperature must be the same for all solutions in a series, including reference solutions. Not over 0.02 milliequivalent of chloride ion may be present since dichromate will be consumed through oxidation of the chloride.

In determination of starch from paper the photometric method by iodine (Vol. II, pp. 231-2), is more reproducible than either the enzymic method or the Tappi method.[32] In particular the photometric method has a consistent blank, one which does not vary with the base paper.

For determination of starch by phenol in sulfuric acid see page 167. For determination by the color in 79% sulfuric acid see page 168.

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[28] G. H. Carter and A. M. Neubert, *J. Agr. Food Chem.* 2, 1070-2 (1954); Cf. D. L. Mould, *Biochem. J.* 58, 593-600 (1954); Cf. J. W. Van Dyk and M. L. Caldwell, *Anal. Chem.* 28, 318-20 (1956); S. Augustat, *Ernäh rungsforschung* 3, 567-74 (1958).

[29] Jules Carles, *Bull. soc. chim. biol.* 36, 705-10 (1954).

[30] K. M. Clegg, *J. Sci. Food Agr.* 7, 40-44 (1956); Cf. Tomoya Nakata, *Tokyo Jikeikai Ika Daigaku Zasshi* 72, 464-70 (1957).

[31] Norman F. Schulz, *Anal. Chem.* 25, 1762-4 (1953).

[32] J. L. Harvey, B. W. Forsher, and D. G. Fletcher, *Tappi* 42, 878-83 (1959).

Samples—*Apples of the jonathan, golden delicious, standard delicious, and winesap varieties.*[33] Grind wedge-shaped slices cut from the opposite sides of the apple. To a 5-gram sample stir in 20 ml. of 1:3 dilution of 72% perchloric acid and let stand 5-10 minutes to disperse starch. Dilute depending upon the starch concentration as follows: 500 ml. for 1.5-3.5% of starch, 250 ml. for 0.6-1.5% of starch, and 100 ml. for a trace-0.7% of starch. Mix, filter through glass wool, and develop an aliquot containing 0.4-2.9 mg. of starch with iodine.

Starches, cereals, cereal by-products, and poultry rations.[34] To a 0.2-gram finely ground sample add 2 drops of 80% ethanol to aid mixing, and 5 ml. of water. Stir and add 25 ml. of hot 80% ethanol. Stir and let stand for 5 minutes. Centrifuge, decant, and repeat the extraction using 30 ml. of hot 80% ethanol. Combine the extracts and evaporate ethanol, which interferes with the anthrone reagent, at reduced pressure to obtain the sugars for analysis.

Add 5 ml. of water to the residue from the ethanol extraction. To dissolve the starches, add 6.5 ml. of 52% perchloric acid prepared by adding 270 ml. of 70% perchloric acid to 100 ml. of water. Stir for 5 minutes and then occasionally during a 15-minute period. Add 20 ml. of water, centrifuge, and decant. Add 5 ml. of water to the residue and repeat the extraction, stirring occasionally during a 30-minute period. Combine the extracts and dilute to 100 ml. with water. Filter, discarding the first 5 ml. of filtrate. Dilute an aliquot so that the final concentration is about 0.1 mg. per ml. Develop with anthrone.

Procedure—*By iodine.* To an aliquot containing 0.4 to 2.9 mg. of starch add 1 ml. of 5% potassium iodide solution and 1 ml. of 0.03567% potassium iodate solution. Allow 5 minutes for the iodine to react with the starch. Dilute to 100 ml., mix, and read at 620 mμ.

By anthrone. As a reagent, dilute 760 ml. of concentrated sulfuric acid to 1 liter and add 1 gram of anthrone. Prepare two blank solutions, each containing 2 ml. of water; three sample solutions each containing 1 ml. of sample solution containing up to 0.3 mg. of starch and 1 ml. of water; and three standard solutions containing 1 ml. of sample solution and 1 ml. of glucose standard solution containing 0.1 mg. of glucose. This latter is an internal standard. To all tubes, add 10 ml. of anthrone reagent, mix, and cover. Heat at 100° for 12 minutes and cool rapidly to room temperature. Read the sample solutions against the standards at 630 mμ. Take an average of the readings and calculate the equivalent quantity of glucose in the sample solution by comparison with the increased absorption due to 0.1 mg. of glucose. Use a conversion factor of 0.9, based on the fact that 0.9 gram of starch yields approximately 1 gram of glucose on hydrolysis. Calculate the percentage of starch in the sample.

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[33] G. H. Carter and A. M. Neubert, *J. Agr. Food Chem.* 2, 1070-2 (1954).

[34] K. M. Clegg, *J. Sci. Food Agr.* 7, 40-4 (1956).

By dichromate oxidation. If the starch is only partially soluble, use a centrifuged cleared solution. To a 25-ml. or less aliquot containing up to 5 mg. of starch add 2.00 ml, of 2.5% potassium dichromate solution in 1:1 sulfuric acid. Dilute to 27.0 ml. and heat with a reference solution in an autoclave at 120° for 30 minutes or at 100° for 2 hours. Cool, dilute to 100 ml., and read at 422-440 mμ.

MUCOPOLYSACCHARIDES

The formation of relatively insoluble complexes between isolated acid mucopolysaccharides and cetyl trimethyl ammonium bromide is the basis for a turbidimetric determination of acid mucopolysaccharides.[35] Depolymerized mucopolysaccharides do not react. If the acid mucopolysaccharide is dissolved in water, rather than in a buffer, less turbidity develops, although the pH of the buffer does not appreciably affect the turbidity.

Hydrolyzed serum filtrate is developed with diphenylamine in acetic-sulfuric acid.[36] Since acid mucopolysaccharides also bind iron, an indirect method for their determination is based on the determination with thioglycollic acid of the remaining unbound iron in a solution of acid mucopolysaccharide and iron.[37] Beer's law is followed for 0.005-0.5 mg.

Procedure—*By cetyl trimethyl ammonium bromide.* Prepare a 0.2 *M* sodium acetate-acetic acid buffer for pH 6 and adjust to contain 0.88% of sodium chloride. Heat 1 ml. of buffer containing 0.01-0.2 mg. of acid mucopolysaccharide and a blank containing 1 ml. of buffer to 37.5° over a period of 10-15 minutes. Add 2 ml. of 0.0025% cetyl trimethyl ammonium bromide solution in 2% sodium hydroxide solution to each. Mix by inversion and read within 10 minutes against the blank at 400 mμ.

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[35] Nicola Di Ferrante, *J. Biol. Chem.* 220, 303-6 (1956).
[36] M. Bonelli, *Sperimentale* 103, 245-53 (1953).
[37] Nicola Di Ferrante, *J. Biol. Chem.* 209, 579-83 (1954).

By thioglycollic acid. Containing 0.05-0.5 mg. of acid mucopolysaccharide. Prepare a buffer as described under the procedure with cetyl trimethyl ammonium bromide. Heat a 1-ml. aliquot containing 0.05-0.5 mg. of acid mucopolysaccharide in the buffer to 37.5°. Add 1 ml. of iron reagent prepared by dissolving 0.648 gram of ferric chloride hexahydrate, 1 gram of mannitol, and 12 mg. of ascorbic acid in 100 ml. of water. Agitate, heat at 37.5° for 10-20 minutes, and immerse in ice for 10 minutes. Centrifuge for 10 minutes, decant, and invert the precipitate for 30 minutes. Dissolve the precipitate in 2 drops of concentrated nitric acid and dilute to 5 ml. with water. To a 0.5-ml. aliquot, add 1 ml. of concentrated ammonium hydroxide and 2 ml. of 2% thioglycollic acid solution. Dilute to 10 ml. with water and mix. Leave in the dark for 30 minutes and read at 540 $m\mu$.

Containing 0.005-0.05 mg. of acid mucopolysaccharide. Follow the procedure for 0.05-0.5 mg. of acid mucopolysaccharide making the following substitutions. Use a 0.2-ml. aliquot, add 0.5 ml. of ferric chloride reagent, and dilute the final volume to 5 ml.

CHITIN

Chitin, a nitrogen containing polysaccharide, is determined by its turbidity in acacia gum solution.[38] The sample is heated with nitric acid for 30 seconds. An increase in the heating time of 5 seconds increases the hydrolysis of 1 mg. of chitin from 4% to 10%. Beer's law is followed by the technic shown for 1-12 mg. of chitin. The method is also applicable to samples containing 0.1-1 mg. of chitin.

Procedure—To a colorless calcium-free sample containing 1-10 mg. of chitin, add 0.3 ml. of concentrated nitric acid and heat at 100° for 30 seconds. Immediately add 5 ml. of 1% gum acacia solution, cool, and neutralize with 30% sodium hydroxide solution. Dilute to 10 ml. with 1% acacia gum solution. Let stand at room temperature for 30 minutes and read at 550 $m\mu$.

PROTEIN-BOUND CARBOHYDRATES

As in the determination of glucose, anthrone can be applied as a reagent for the determination of mannose and galactose for estimation of protein-bound carbohydrates.[39] In the presence of tryptophan, a 530 $m\mu$

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[38] Juliusz Popowicz, *Chem. Anal.* (*Warsaw*) **4,** 7-8 (1959).

[39] E. F. Tuller and N. R. Keiding, *Anal. Chem.* **26,** 875-8 (1954).

absorption peak appears. This is caused by a complex formed by the interaction of the sugar, sulfuric acid, and excess reagent. To eliminate the tryptophan interference it is possible to acid-hydrolyze the material before analysis or to use a nomogram prepared by plotting the ratio of the height of the 530 mμ peak to that of the 630 mμ peak against the 630 mμ reading to correct for the tryptophan interference. The use of the nomogram may cause low values in the carbohydrate determination. Desoxycorticosterone acetate, glucosamine, cupric ion, ferrous and ferric ions, and pentoses do not produce this 530-mμ band in sugar determinations with anthrone. Addition of boric acid to sulfuric acid and tryptophan as a reagent (Vol. III, p. 233) improves the clarity of color as a pure violet.[40]

Procedure—*Nonhexosamine carbohydrate protein precipitates.* Add 0.5 ml. of a 1:10 dilution of serum to 10 ml. of 95% ethanol. Let stand for 10-15 minutes and centrifuge. Dissolve the precipitate in 10 ml. of 4.4% sodium carbonate solution. To 2 ml. of sample solution containing 0.12 mg. of galactose and mannose add 0.5 ml. of 1% anthrone solution in ethyl acetate which has been aged at least 4 hours, preferably over night. Carefully layer 5 ml. of concentrated sulfuric acid into the tube and swirl rapidly to hydrolyze the ethyl acetate and mix the contents. Let this stand for 30-90 minutes for maximum color development and read at 530 and 630 mμ. Read the carbohydrate by the nomogram shown in Figure 10.

Total serum protein. Follow the procedure under nonhexosamine carbohydrate ending with ". . . and centrifuge." Discard the precipitate and develop an aliquot of the upper layer.

Total protein in precipitates. To 2 ml. of the protein precipitate dissolved in 4.4% sodium carbonate solution add 2 ml. of 2:1 hydrochloric acid and reflux for 4 hours. Dilute the hydrolysate to 10 ml. and develop 2 ml. of the dilution.

METHYLCELLULOSE

Methylcellulose develops a color when heated in an acid solution of diphenylamine.[41] The maximum absorption at 640 mμ follows Beer's law in the range of 0.01-0.1 mg. per ml. This has the advantage over the

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[40] J. Badin, C. Jackson, and M. Schubert, *Proc. Soc. Exptl. Biol. Med.* 84, 288-91 (1953).

[41] G. Kanzaki and E. Y. Berger, *Anal. Chem.* 31, 1383-5 (1959).

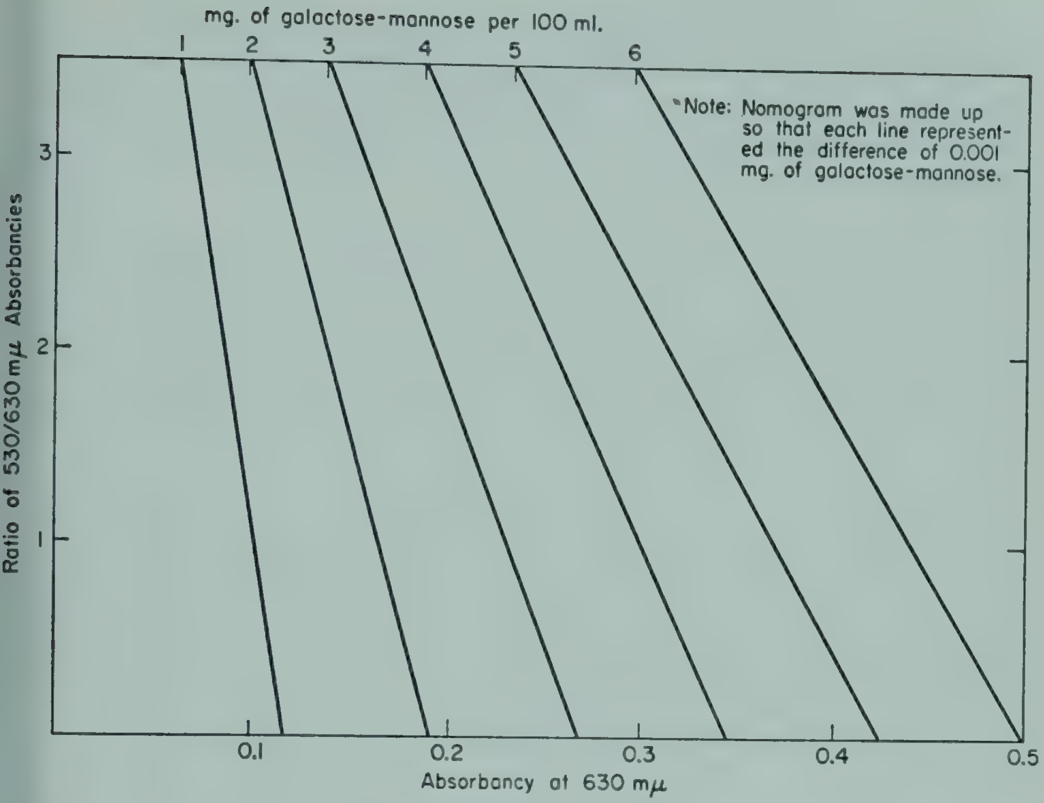

FIG. 10

Nomogram for determination of carbohydrates in the presence of tryptophane

anthrone technic for methylcellulose in not producing enough heat to cause color development, thereby eliminating the need for controlling the heat upon addition of the reagent. The temperature used to heat the reaction mixture, however, should be constant. The actual degree of heat is not critical but its constancy is. Proteins are removed by precipitation with zinc.

Procedure—If protein is present, add 1 ml. of 10% zinc sulfate septihydrate solution in 1:143 sulfuric acid to a diluted sample containing 0.02-0.08 mg. methylcellulose per ml. Precipitate the protein by adding 1 ml. of 3% sodium hydroxide solution and let stand for at least 15 minutes. Centrifuge and use the supernatant liquid. Avoid the use of filter paper because it adds a blank.

To a 2-ml. sample containing 0.02-0.08 mg. of methylcellulose per ml., add 5 ml. of reagent prepared by dissolving 3.75 grams of colorless diphenylamine crystals in 150 ml. of glacial acetic acid and adding 90 ml.

of concentrated hydrochloric acid. Mix, cover, and heat in an oil bath at 108° for 30 minutes. Cool rapidly for 10 minutes and read at 640 mμ against a blank containing 2 ml. of water and 5 ml. of reagent similarly heated. If the blank reads less than 90% transmittance, the reagent has deteriorated.

BETA- AND GAMMA-CELLULOSE

Gamma-cellulose is soluble in 18% sodium hydroxide and not precipitated on neutralization to pH 6. Beta-cellulose is similarly soluble in 18% alkali but precipitated at pH 6. By suitable manipulation both are determined by the chromic ion formed on oxidation by potassium dichromate.[42]

Procedure—Weigh 2 grams of finely dissolved pulp calculated on a moisture-free basis. Add 100 ml. of 18% sodium hydroxide solution. Add glass beads to disperse lumps of fibers and shake for 5 minutes at 23°. Store for 1 hour at 25° and filter on a Gooch or fritted-glass crucible. Refilter without drawing air through the mat of fibers.

Titrate a portion with 1:4 sulfuric acid to a methyl red end point. From these data similarly acidify a 20-ml. portion without indicator. Add 25 ml. of 4.9% potassium dichromate solution and dilute to 75 ml. Add 100 ml. of concentrated sulfuric acid with swirling. After 15 minutes cool and dilute to 200 ml. Read at 590 mμ. Report as beta- plus gamma-cellulose from a curve developed with glucose.

Acidify a 40-ml. portion of the liquor based on the earlier titration and shake vigorously. Let it stand for several hours or freeze to coagulate the beta-cellulose. Dilute to 100 ml. and centrifuge. Pipet out a 50-ml. portion and oxidize as before from "Add 25 ml. of 4.9%. . . ." This time the result is gamma-cellulose. Obtain the beta-cellulose by difference.

CELLULOSE RESINS

Cellulose ethers such as methyl and ethylcellulose and cellulose ethers such as cellulose acetate, propionate, and acetate-butyrate are determined by the blue-green color formed with anthrone.[43] Plasticizers and other resins do not interfere, but nitrocellulose should be absent. The color development is influenced by the type of cellulose resin present. Not all cellulose resins yield the same amount of color. The color is stable for 24 hours, and is effected by variations in concentration of acid, heating time, temperature, and size and quality of tubes used. Ethylcellulose can be extracted with a methanol-water solvent from a light mineral oil suspension prior to determination by anthrone. Similarly, a methanol-acetone solvent can be used for cellulose acetate butyrate.[44]

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[42] Ralph M. Kingsbury, *Tappi* 37, 353-4 (1954).

[43] M. H. Swann, *Anal. Chem.* 29, 1505-6 (1957).

Procedure—*By anthrone. Cellulose resins in general.* To prepare the reagent, warm a 0.5% anthrone solution in absolute ethanol at 70°, agitating frequently. Weigh a sample, dissolve in acetone, and dilute so that a 1-5 ml. aliquot contains up to 0.5 mg. of nonvolatile resin. If solution is not complete dioxane may be added. Dry an aliquot at 105° in an oven and add 10 ml. of cooled 3:1 sulfuric acid and 0.5 ml. of 0.5% anthrone solution in absolute ethanol. Stopper, agitate, and heat at 90° for 20 minutes with occasional agitation. Cool to room temperature and read at 625 mμ against a blank similarly treated.

Ethylcellulose from benzene. Dilute an aliquot of sample containing 0.005-0.05 mg. of ethylcellulose dissolved in benzene to 10 ml. with benzene. Heat in a 90° glycerol bath to evaporate the solvent. Add 2 ml. of 3:1 sulfuric acid and warm to dissolve the dry ethylcellulose film. Add 8 ml. of water and cool in water. Add 20 ml. of 0.2% anthrone solution in concentrated sulfuric acid, at least 4 hours and not over 12 hours old. Mix and heat at 90° for 16 minutes with frequent stirring. Cool and read at 625 mμ against a benzene blank.

Ethylcellulose from light mineral oil. Dilute an aliquot containing 0.005-0.05 mg. of ethylcellulose in 1:9 benzene-mineral oil to 10 ml. with 1:9 benzene-mineral oil mixture. Prepare a standard blank of 10 ml. of the benzene-mineral oil mixture.

Add 5 ml. of 19:1 methanol-water solvent to the standard blank, and sample and stopper. Shake and centrifuge to extract the ethylcellulose. Decant the methanol-water layer and repeat the extraction 4 times. Heat the combined extracts at 90° to evaporate the solvent and follow the procedure as the ethylcellulose in benzene starting with "Add 2 ml. of 3:1 sulfuric acid. . . ."

Cellulose acetate-butyrate from ethylene dichloride. Dilute an aliquot containing 0.005-0.05 mg. of cellulose acetate-butyrate in ethylene dichloride to 10 ml. with ethylene dichloride. Prepare an ethylene dichloride blank containing 10 ml. of solvent. Treat the blank and sample following the procedure for ethylcellulose in benzene starting with "Heat in a 90° glycerol bath. . . ."

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[44] E. P. Sansel and J. C. Aldrich, *Ibid.* 29, 574-6 (1957).

Cellulose acetate-butyrate from mineral oil. Dilute an aliquot containing 0.005-0.05 mg. of cellulose acetate-butyrate in a 1:9 ethylene dichloride-mineral oil mixture to 10 ml. with 1:9 ethylene dichloride-mineral oil solvent. Prepare a 10-ml. blank of the 1:9 ethylene dichloride-mineral oil solvent. Follow the procedure for ethylcellulose in mineral oil starting at "Add 5 ml. of 19:1 methanol-water solvent. . . ."

CHAPTER 10

GLUCOSIDES

THE GLYCOSIDES yield pentose or hexose sugars on hydrolysis. Therefore many of the methods of determination are those for the sugars. For determination of methyl-glucoside by *N*-ethyl carbazole see page 168. For the determination of digitoxigenin or gitoxigenin by 2,4-dinitrophenylsulfone see page 233.

Alcoholic solutions of strophanthidin and its glucosides produce a yellow color with concentrated sulfuric acid which follows Beer's law. The reaction is dependent upon the —CHO group on C-10 and is specific for the genin but the position and magnitude of absorption maximum are dependent on the sugar portion of the glycosides.[1]

For determination of methyl-2,3,5-tri-*O*-methyl-*l*-arabinoside by phenol in sulfuric acid see page 167. For determination of adenosine, adenine riboside by anthrone see page 194. For determination of methyl-2,3-di-*O*-methyl-*d*-xyloside or 2,3,5-tri-*O*-methyl-*l*-arabinose by phenol in sulfuric acid see page 167.

Hesperidin and eriodictin are glucosides present in citrin. Both give the indophenol reaction with a maximum absorption at 620 mμ.[2] After hydrolysis of citrin the two are separable by paper chromatography. Water saturated with benzene is used for development by the one-dimensional ascending method.

CEREBROSIDES

Cerebrosides are determined on the basis of their galactose content with orcinol (Vol. III, pp. 239-40). The cerebrosides are extracted from gangliosides in nervous tissue with chloroform in the presence of 0.1% saline solution. Lipides are extracted with a chloroform-methanol mixture. Beer's law is followed for 0.01-0.1 mg. carbohydrate.[3]

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[1] S. Goldschmidt, B. Keober, and E. Helmreich, *Hoppe-Seyler's Z. physiol. Chem.* **290**, 106-19 (1952).

[2] Konosuke Murai, *Yakugaku Zasshi* **78**, 745-8 (1958).

[3] L. Svennerholm, *J. Neurochim.* **1**, 42-53 (1956); E. Svennerholm and L. Svennerholm, *Scand. J. Clin. and Lab. Invest.* **10**, 97-101 (1958).

Samples—*Nervous tissue.* Homogenize, lyophilize, and grind the sample. Reflux in a micro-Soxhlet apparatus for 2 hours with a 2:1 mixture of chloroform and methanol to extract lipids. Filter and wash the flask and filter paper with an equal volume of chloroform. Combine the washings and filtrate and add one-half volume of 0.1% saline solution. Mix by shaking for 1 minute and allow to stand for 48 hours. Remove the upper layer and wash the lower layer three times with the upper phase from a mixture of 800 ml. of 2:1 chloroform-methanol and 200 ml. of 0.1% saline solution.

Dry a portion of the sample solution containing 0.01-0.1 mg. of galactose under nitrogen at 50° and hydrolyze for 2 hours at 100° with 0.5 ml. of ethanol and 2 ml. of 1:11 sulfuric acid. Filter and develop.

Plasma. Lyophilize a 10-ml. sample with 5 ml. of 0.2 *M* phosphate buffer for pH 6, Vol. I, p. 174. Extract the residue with 50 ml. of a boiling 2:1 mixture of chloroform and methanol followed by three extractions with 10-ml. portions of the same mixture. Remove the solvent *in vacuo* and take up in 10 ml. of solvent. Dilute to 20 ml. and extract first with 5 ml. of 0.1% sodium chloride solution saturated with the solvent mixture followed by 2 ml. of the same sodium chloride solution. Discard the saline extracts and evaporate the organic solution to dryness at 40°. Take up in 0.5 ml. of ethanol and 2 ml. of 1:11 sulfuric acid. Heat for 30 minutes at 100° and filter. Wash the tube and filter with 1:11 sulfuric acid and dilute the washings and filtrate to 10 ml. with 1:11 sulfuric acid.

Procedure—Treat an aliquot of sample containing 0.01-0.1 mg. of galactose chilled to 0° with 0.2% orcinol solution in concentrated sulfuric acid which has been chilled to 0°. Chill the reaction mixture to 0° and read at 505 mμ. (Cf. Vol. III, p. 240.)

Digitoxin

Some relative idea of the sensitivity of various reactions for digitoxin and related drugs can be obtained from Table 15,[4] recognizing that the sensitivity of different reagents varies somewhat with changes in detail of the technic. The 2,4-dinitrophenylsulfonic acid reagent is most sensitive in each case.

The official USP method depends on a chromatographic separation in the presence of formamide and development by picric acid.[5] 2,4-Dinitro-

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[4] D. H. E. Tattje, *J. Pharm. and Pharmacol.* 10, 493-8 (1958).

[5] U. S. Pharmacopoeia, fifteenth ed. pp. 217-8 Mack Publishing Co. Easton Pennsylvania (1955).

TABLE 15. MAXIMUM REPORTED SENSITIVITY OF VARIOUS METHODS OF DETERMINATION OF DIGITOXIN AND RELATED DRUGS EXPRESSED AS MOLAR EXTINCTIONS

Glycoside or aglycone	*Treatment*	*Reagent*			
		2,4-Dinitro-diphenyl-sulfone	*Picric acid*	*3,5-Dinitro-benzoic acid*	M-*dinitro-benzene*
Digitoxin	None	23,200	17,000	8500	14,700
	Boiled with acid	28,300	17,600	8140	. . .
Digitoxiginin	None	24,400	17,200	9540	10,720
	Boiled with acid	29,100	18,200	12,300	. . .
Gitoxin	Boiled with acid	18,100	15,600	7810	. . .
Gitoxiginin	None	. . .	13,500	7290	3920
	Boiled with acid	18,100	14,800	11,100	. . .

phenylsulfone as a reagent for digitoxin, digitoxigenin, and gitoxigenin, produces a color which is read at 600 mμ rather than at 500 mμ where there is interference from the reaction of sodium hydroxide with the yellow pigment digitoflavone in digitalis leaves.[6] The corresponding sulfide and sulfoxide of the reagent do not react with the digitalis glucosides. The extinction of digitoxin and digitoxinin is substantially increased by preliminary boiling with acid.

Gitoxigenin gives a straight line curve only if pretreated with acid. Digitoxin and digitoxigenin produce a straight line curve whether pretreated with acid or determined directly without pretreatment. The concentration of sodium hydroxide influences the color density and speed of reaction, a concentration of 6% being optimal. The maximum time for full color development after addition of sodium hydroxide is 5.5 minutes for digitoxin, 4.5 minutes for digitoxigenin, and 3 minutes for both these compounds and for gitoxigenin after acid treatment. The color is constant for 1-1.5 minutes. Temperatures higher than 20° should be avoided during the reaction.

The action of *m*-dinitrobenzene on cardiac glycosides (Vol. III, p. 243) and aglycones, such as convallatoxin, deacetylanhydro-oleandrin, dianhydrogitoxigenin, and digitoxin, produces a two-stage reaction, a red color in the presence of dilute sodium hydroxide or an alkaline buffer at pH 11-13 followed by a blue color in the presence of more concentrated alkali.[7] Since the blue color is labile, the determination of cardiac glycosides by the blue color requires a low temperature and knowledge of the rate of decomposition. Since the red color is more stable and allows the determination of the glycosides at room temperature, a method has been developed utilizing the first reaction of the glycoside with the reagent. The color is different than that given by simple ketones such as acetone and dehydro*iso*androsterone. A higher concentration of alkali produces a more intense color but the rate of fading is more rapid. Beer's law is followed and it is not necessary to apply a correction for reagents. The use of pyridine in place of ethanol increases the sensitivity by 3-7 times.[8]

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[6] D. H. E. Tattje, *J. Pharm. and Pharmacol.* **10,** 493-8 (1958).

[7] C. H. Hassall and A. E. Lippman, *Analyst* **78,** 126-7 (1953).

Ferric ion and sulfuric acid (Cf. Vol. III, p. 243) are reagents for the digitoxin component of digitalis preparations, in the presence of cholesterol.[9] When developed by this reaction, acetyldigitoxin, lanatoside C, and *k*-strophantoside have the same maximum absorption.

Digitalis glucosides give a color with 3,5-dinitrobenzoate in methanol, cf. preceding method. The digitoxin gives a maximum color after 5 minutes, digitoxigenin and gitoxigenin treated with acid in 4 minutes, and the latter two untreated in 3 minutes.[10] The reaction of digitoxin with phosphoric acid includes digitoxose.[11] The reaction with 3,5-dinitrobenzene sulfonic acid includes digitoxin, strophanthin, and ouabain.[12] Various glucosides of the digitoxin class react with picric acid [13] (Vol. III, p. 244).

Xanthydrol produces a vivid red color with the digitosose molecule of the digitalis glucosides if the alcoholic basic digitoxin solution containing 0.5 mg. per ml. is heated in glacial acetic acid containing 1% of hydrochloric acid at 100°.[14] The glucose part of the molecule can be determined by anthrone.[15]

Procedure—*By picric acid.* As reagent mix 20 ml. of a 1% solution of picric acid with 10 ml. of 5% sodium hydroxide solution. Dilute to 100, mix, and use within 2 days.

Dissolve a sample containing about 20 mg. of digitoxin in ethanol and dilute to 50 ml. with the same solvent. Mix a 5-ml. aliquot with 1 gram of USP purified silicious earth and evaporate to dryness. Mix with 2 ml. of water.

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[8] Kurt Repke, *Arch. exptl. Pathol. Pharmacol.* 234, 147-63 (1958).

[9] S. Magalini, G. A. Cinotti, and G. De Simone, *Farmaco, (Pavia) Ed. sci.* 10, 510-23 (1955); G. A. Cinotti, G. De Simone, and S. Magalini, *Giorn. biochim.* 5, 152-8 (1956).

[10] D. H. E. Tattje, *Pharm. Weekblad* 91, 841-6 (1956); J. Lemli, *Pharm. Tijdschr. Belg.* 34, 157-64 (1957).

[11] R. Dequeker, *Verhandel. Koninkl. Akad. Geneesk. Belg.* 15, 335-51 (1953).

[12] Masami Akatsuka, *Yakugaku Zasshi* 78, 62-4 (1958).

[13] Julia Luna Seminario, *Anales fac. farm. y bioquim., Univ. nacl. mayor San Marcos (Lima, Peru)* 1, 362-76 (1950).

[14] P. Soos, L. Virf, and A. Blazsek, *Orvosi Szemie* 2, 67-72 (1956).

[15] Tomoya Nakata, *Tokyo Jikeihai Ika Daigaku Zasshi* 72, 456-9 (1957).

To purify formamide shake with 10% by weight of anhydrous potassium carbonate for 15 minutes and filter. Distil at under 25-mm. pressure. After it reaches a boiling point of 115° at 25 mm. or 101° at 12 mm. collect the distillate. Store over sulfuric acid until no odor of ammonia is detectable.

Add 4 ml. of formamide to the aqueous sample and heat at 50-60° for 20 minutes with stirring. Cool and add 7 grams of purified silicious earth in portions, stirring until uniform. Prepare a 25 × 200 mm. chromatographic column closed with glass wool and having cotton above it. Charge with about 2 grams of purified silicious earth moistened with 1 ml. of water and tamp down to 15-20 mm. Mix about 3 grams of the sorbant with 2 ml. of formamide diluted with 1 ml. of water. Press this down lightly. Add the sample in sorbant to the top of the column, using three 1-gram portions of sorbant to facilitate quantitative transfer. Top the 120-150 mm. column with cotton.

Moisten the column with 30 ml. of isooctane, using air pressure if required. Supply a 500-ml. separatory funnel to act as a constant-level reservoir. Pass 120 ml. of isooctane through the column. As eluant wash 150 ml. of chloroform with four successive 250-ml. portions of water and filter. Mix 100 ml. with 300 ml. of benzene. Wash with 30 ml. of 2:1 formamide-water and discard the washings.

After the isooctane has passed through, elute the column with 10 ml., 10 ml., and 270 ml. of the eluant mixture. Dilute to 300 ml. Evaporate 30 ml. of eluate to dryness in a stream of air. Moisten with 0.5 ml. of 95% ethanol and again take to dryness. Cool and take up in 5.0 ml. of ethanol. After 15 minutes add 3.0 ml. of the picric acid reagent protected from strong light. Read at 495 mμ against a blank every 2 minutes until a maximum is reached.

By 2,4-dinitrodiphenylsulfone. Without boiling. Dissolve the sample in 4 ml. of 95% ethanol and add 5 ml. of 0.075% solution of dinitrodiphenylsulfone in 95% ethanol. Add 1 ml. of 0.6% sodium hydroxide solution. When the maximum color intensity is reached, read at 600 mμ against a reagent blank. Maintain the temperature at 20°.

With boiling. Dissolve the sample in 5 ml. of 95% ethanol, 2 ml. of 1:2 hydrochloric acid, and 13 ml. of water. Reflux for 30 minutes and wash the reflux condenser and flask twice with 5-ml. portions of 25% ethanol. Shake the combined acid solution and washings with three 22.5-ml. portions of chloroform for 1 minute each. Dry the combined chloroform extracts with anhydrous sodium sulfate and filter. Wash the filter and flask with 5 ml. of dry chloroform and distil the solvent. Dissolve the residue in 4 ml. of 95% ethanol and complete as for samples without boiling.

By m-dinitrobenzene. Treat a 0.1% solution of the glycoside in absolute ethanol with 0.3 ml. of 2% *m*-dinitrobenzene solution in absolute ethanol. Dilute to 1.2 ml. with absolute ethanol and add 0.10 ml. of 0.4% sodium hydroxide solution. Read at 560 mμ after 10 minutes.

By ferric ion and sulfuric acid. Cholesterol absent. To a 0.5-ml. aliquot containing 0.005-0.025 mg. of digitoxin in 95% ethanol add 3 ml. of glacial acetic acid. Add 2 ml. of a reagent prepared by mixing 1 ml. of 10% ferric acetate solution in acetic acid with 99 ml. of concentrated sulfuric acid. Read against a reagent blank at 470 mμ.

Cholesterol present. Follow the procedure for cholesterol absent and read at 470 mμ and 560 mμ.

$$\text{digitoxin} = 0.00414\, D_{470} - 0.00285\, D_{560}/0.0000390107$$

$$\text{cholesterol} = 0.00171\, D_{470} - 0.0106\, D_{560}/0.0000390107.$$

By methyl-3,5-dinitrobenzoate. To a 4-ml. sample in methanol add 5 ml. of 1.5% methyl-3,5-dinitrobenzoate solution in methanol and 1 ml. of 1.8% sodium hydroxide solution. Read at 535 mμ after 5 minutes.

By phosphoric acid. Treat a 1-ml. sample containing 0.002-0.004 mg. of digitoxin in acetone with 4 ml. of 85% phosphoric acid. Maintain at 35° for 15 minutes, cool, and read at 474 mμ.

By 3,5-dinitrobenzenesulfonate. Mix a 4-ml. aliquot containing up to 0.04 mg. of digitoxin in methanol with 0.5 ml. of 2% potassium 3,5-dinitrobenzenesulfonate solution, and 0.5 ml. of 5% potassium hydroxide solution. Let stand for 10 minutes at room temperature and read at 530 mμ.

By 3,5-dinitrobenzoic acid. Tincture. Dilute a 1-ml. sample with 9 ml. of 70% ethanol. Add 15 ml. of water and filter. Mix the filtrate with 10 grams of active aluminum oxide. Filter and reflux a 5-ml. aliquot with 5 ml. of water and 1 ml. of 1:15 sulfuric acid for 1 hour. Add 2 grams of barium carbonate and filter on an inorganic filter. To a 5-ml. aliquot add

2 ml. of 2% 3,5-dinitrobenzoic acid solution in ethanol, 1 ml. of 4% sodium hydroxide solution, and 2 ml. of 0.25% ethylenediaminetetraacetate solution in 50% ethanol to eliminate interferences. Read against a reagent blank.

General.[16] Stir 0.7 gram of drug with 70 ml. of water at 90° for 15 minutes. Cool rapidly and bring back to 70 grams plus the weight of sample with water. Filter, discarding the first 10 ml. Mix 50 ml. of filtrate, 30 ml. of water, and 5 ml. of 1:5 hydrochloric acid. Simmer for 15 minutes and cool. Neutralize with 1:5 ammonium hydroxide.

Extract with four successive 20-ml. portions of ether. Dry the combined extracts with 2 grams of calcium oxide and 3 grams of anhydrous sodium sulfate. Separate the ether layer and wash the drying agent with 15 ml. of ether. Evaporate the filtrate and washings. Take up the residue in 10 ml. of 95% ethanol. Dilute 2 ml. of the solution to 5 ml. with 95% ethanol, 9 ml. of 95% ethanol, and 2 ml. of 4% sodium hydroxide solution. Dilute to 25 ml. with water and read at 560 mμ after 5 minutes at 20°.

DIGOXIN

Digoxin is assayed by its reaction with *m*-dinitrobenzene at 0-2° and read 5 minutes after addition of alkali to develop the color.[17] Gitoxin yields a similar color and must be absent. By lowering of the concentration of alkali the color is more stable and up to 8% of gitoxin does not interfere.[18] Then at 22-25° the maximum color is attained in 25-30 minutes and remains within 2% of the maximum for five minutes. Deviation from Beer's law is negligible at 0.1-0.3 mg. The alkali concentration is critical so anything which reacts with it interferes.

Samples—*Injection solutions.* Mix a volume of sample equivalent to 2.5 mg. of the glycoside with 50 ml. of 10% sodium sulfate solution and 2 ml. of 1:10 hydrochloric acid. Extract with seven 25-ml. portions of chloroform. Wash each chloroform extract with 10 ml. of water and filter through cotton. Evaporate the combined chloroform extracts to dryness and take up the residue in hot 95% ethanol. Cool and dilute to 50 ml. with the same solvent.

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[16] W. Hauser and I. Mathauser, *Sci. Pharm.* 26, 258-62 (1958).

[17] U. S. Pharmacopoeia, fifteenth ed., pp. 222-3, Mack Publishing Company, Easton, Pennsylvania (1955).

[18] Daniel Banes, *J. Am. Pharm. Assoc.* 43, 355-7 (1954)

Tablets. Mix a portion of ground tablets equivalent to 2.5 mg. of digoxin with 20 ml. of 95% ethanol. Simmer for 20 minutes with frequent stirring. Use three 10-ml. portions of 95% ethanol for transfer for extraction. Add 50 ml. of water and cool. Add 1 ml. of 1:10 hydrochloric acid and extract at once with 50 ml. and 50 ml. of isooctane. Extract the combined isooctane extracts with 20 ml. of 50% ethanol followed by 20 ml. and 20 ml. of water. Discard the isooctane and combine the three extracts. Dilute to about 400 ml. with 10% sodium sulfate solution. Proceed as for injection solutions from "Extract with seven. . . ."

Procedure—*USP.* Transfer 5 ml. of sample and 5 ml. of standard to tubes. To each add 5 ml. of fresh 1% *m*-dinitrobenzene solution in 95% ethanol. Cool to 0-2°. Add to each 2 ml. of 20% sodium hydroxide solution recording the time. Read each exactly 5 minutes later at 620 mμ against a reagent blank.

Modified. As reagent dissolve 1.6 grams of *m*-dinitrobenzene in 80 ml. of hot 95% ethanol. Cool, add 1 ml. of 10% tetramethylammonium hydroxide solution, and dilute to 100 ml. with 95% ethanol. Evaporate 5 ml. of prepared sample and a parallel standard to dryness at 100°. Cool in a vacuum desiccator. Add 4 ml. of freshly prepared reagent and mix frequently for 15 minutes at room temperature. Read at 615 mμ against a reagent blank until a maximum color is attained.

Gitoxin

The color developed by gitoxin with concentrated sulfuric acid containing a trace of ferric ion is appropriate for its estimation.

Procedure—The sample solutions were prepared for determination of digoxin, page 235. Evaporate 20 ml. of the prepared sample to dryness. Add 2 ml. of 1:1 95% ethanol-concentrated hydrochloric acid. Stir occasionally at room temperature during the next 30 minutes. Add 10 ml. of 50% ethanol and simmer gently for 30 minutes. Add small amounts of 95% ethanol to maintain the volume at 10-12 ml. Cool and add 100 ml. of water. Extract with three 20-ml. portions of chloroform. Wash each chloroform extract with 10 ml. of water. Filter the combined chloroform extracts through cotton and evaporate to dryness.

Take up the residue by heating with 5 ml. of isooctane. Cool and chromatograph the turbid mixture on a 10-gram Celite column using 5 ml. of 50% ethanol as the immobile solvent and isooctane as the mobile solvent.[19] Discard the isooctane used in preparing the column. Discard a forerun of 50 ml. and collect the next 70 ml. of isooctane. Evaporate to dryness and cool. Add 1 ml. of a mixture of 100 ml. of concentrated sulfuric acid with 0.04 ml. of 9% ferric chloride solution. Read at 530 mμ against a reagent blank.

OUABAIN, *g*-STROPHANTHIN

g-Strophanthin is commonly found with invert sugar in injection solutions. It can be determined by evaporation, ethanol extraction, and development with alkaline picrate.[20] As a preferable technic the *g*-strophanthin is extracted with ethanol from the aqueous solution saturated with potassium carbonate. The result differs by no more than 3% from the value for strophanthin without invert sugar present.

Determine ouabain by the method by color reaction with *m*-dinitrobenzene, page 234, but carry out the reaction at 0° in the presence of piperidine instead of alkali to give a pink color which turns to a stable orange-red. For ouabain by picric acid see page 232. For determination by 3,5-dinitrobenzenesulfonic acid see page 234.

Procedure—Mix 5 ml. of the aqueous sample with 10 ml. of absolute ethanol. Add potassium carbonate until the solution is saturated and excess is present. Shake for 2 minutes and allow the layers to separate. Separate the ethanol phase and add 2 ml. of water. Again saturate with potassium carbonate and shake for 2 minutes. Separate the ethanol layer and dilute to 10 ml. with absolute ethanol.

To 5 ml. of the slightly turbid ethanol layer add 5 ml. of 0.25% aqueous picric acid solution and 1 ml. of 2.5% aqueous sodium hydroxide solution. After 10 minutes read at 494 mμ against a reagent blank.

k-STROPHANTHIN

The technic with picric acid is applicable to *k*-strophanthin in injection solutions with procaine.[20] Strophanthin is also determined by 3,5-dinitrobenzenesulfonic acid, page 234.

Procedure—To 5 ml. of the aqueous solution add 1 ml. of 2.5% aqueous sodium hydroxide and 5 ml. of 1% solution of picric acid in 95% ethanol. Read at 494 mμ after 5 minutes against a reagent blank.

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[19] For an example of handling of such a column see page 233.

[20] J. Richter, *Pharmazie* **9,** 986 (1954); **11,** 390-1 (1956).

PHLORIZIN

Phlorizin produces a red color with hydrogen peroxide which follows Beer's law for 0.01-1 mg. per ml. at pH 9. Since the rate of the reaction depends on the pH and the final product acts as an indicator, green at pH 12, red at pH 9 and yellow at pH 6.5, a borate buffer is used to maintain the pH at 9.[21] Samples are extracted with butanol to prevent interference from electrolytes. Hemolysis in plasma samples does not affect results.

Glucose and ascorbic acid in concentrations likely to be found in biological samples do not interfere since they are not extracted. Phloretin, phlorin, and phloretic acid do not interfere. Insofar as phloroglucinol is extracted into the butanol layer and back into the buffer it will interfere.

Procedure—*Plasma.* To a 4-ml. sample, add 5 ml. of butanol, and one drop of concentrated nitric acid. Shake for 3 minutes and separate the emulsion formed by centrifuging for 10 minutes. Add 3 ml. of the butanol layer to 5 ml. of borate buffer at pH 9.1 prepared by mixing 9 parts of 3.8% disodium tetraborate decahydrate (borax) solution, with 1 part of 2.5% boric acid solution. Shake for 3 minutes and separate the aqueous layer.

To 2 ml. of the aqueous layer containing 0.02-2 mg. of phlorizin, add 1 ml. of 0.1% hydrogen peroxide solution. Let stand for 2 hours and read at 490 mμ. Subtract a water blank.

Bile. Add a 1-ml. sample to 14 ml. of water. Decolorize by adding 2 ml. of 2.5% barium hydroxide solution and 2 ml. of 5% zinc sulfate heptahydrate solution. Shake and filter. Add 5 ml. of butanol and one drop of concentrated nitric acid to 5 ml. of the filtrate. Shake and separate the butanol layer. Follow the procedure for plasma, starting at "Add 3 ml. of the butanol layer. . . ."

Urine. Prepare a 5-ml. sample following the procedure for plasma, omitting the centrifuging.

RHAPONTIN, RHAPONTICIN, 4-METHOXY-3,3′,5-STILBENETRIOL 3-GLUCOSIDE

This is the characteristic glucoside of rhubarb, separated by descending paper chromatography and developed with vanillin.[22] Alternatively it is developed with potassium ferricyanide and ferric ammonium sulfate.[23]

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[21] F. A. Jenner and D. H. Smyth, *Biochem. J.* **71,** 138-140 (1959).

[22] E. W. Neuhoff and H. Auterhoff, *Deut. Apoth. Ztg.* **94,** 541-4 (1954).

[23] M. Henneberg and P. Horak, *Acta Polon. Pharm.* **16,** 189-96 (1959).

Sample—Reflux 0.5 gram of finely ground pharmaceutical rhubarb with 10 ml. of 95% ethanol for 15 minutes. Decant and reflux the residue with 7.5 ml. and 7.5 ml. of 95% ethanol for 10 minutes. Dilute to 25 ml. with 95% ethanol and chromatograph a 0.2-ml. portion by descending paper chromatography using chloroform-methanol-acetic acid 3:2:0.5, the whole being saturated with water. An alternative is ethanol-water-butanol. Digest the spots of rhaponticin with 5 ml. of 0.4% sodium hydroxide solution and develop by ferricyanide and ferric ion.

Procedure—*By vanillin.* Chill 5 ml. of approximately 0.1% solution. Add 10 ml. of chilled 0.2% solution of vanillin in concentrated sulfuric acid. Read the deep red color.

By ferricyanide and ferric ion. To 5 ml. of sample in 0.4% sodium hydroxide solution add 1 ml. of 0.2% solution of potassium ferricyanide and 1 ml. of 0.06% solution of ferric alum in 1:70 sulfuric acid. Read the blue color.

RUTIN, VITAMIN P, 5,7,3′,4′-TETRAHYDROXFLAVONOL-3-RHAMNOGLUCOSIDE

Rutin can be measured directly after extraction from the sample with acidified ethanol.[24] Depending on the pH and solvent there are maxima at 255 mμ and 352.5 mμ. More than 5% of quercetin interferes. Rutin decomposes in strongly alkaline media. Beer's law is followed. Under controlled conditions of alkalinity a yellow color is read.[25]

Either aluminum chloride (Vol. III, pp. 249-50) or aluminum nitrate[26] provide the aluminum ion for estimation of rutin in buffered solution by a red color. Quercetin is extracted from rutin by diethyl ether. Interfering colored compounds are precipitated from hot aqueous solution by an alkaline solution of plumbous ion.[27] Rutin, the glucoside, on hydrolysis yields quercitin (page 146) and a sugar. Many of the reactions are similar. Therefore many of the problems relate to separation.

Quercitin and rutin are sorbed on an aluminum oxide column. Elution with methanolic hydrochloric acid saponifies the rutin to quercitin.[28] Thereafter it is reduced in hydrochloric acid solution by magnesium amalgam or zinc amalgam. The resulting red color has its maximum at about 530 mμ.

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[24] Arthur Turner, Jr., *J. Assoc. Offic. Agr. Chemists* **36,** 699-707 (1953).

[25] A. A. Konstantinov, *Sbornik Nauch. Rabot i Avtoreferat. Kharbarovsk. Inst. Epidemiol. i Gigieny* **1951-55,** No. 3, 159-60.

[26] K. Imai and K. Furuya, *J. Pharm. Soc. Japan* **72,** 1560-4 (1952); K. Kakemi, T. Uno, and M. Iwama, *Ibid.* **73,** 101-2 (1953); J. Gierlowska, S. Kwiatkowska, and A. Reinheres, *Nahrung* **2,** 705-9 (1959).

[27] Walter Brune, *Arch. Pharm.* **287,** 432-9 (1954).

Of the complexes of quercitin and rutin with zirconium, aluminum, titanium, copper, and cadmium, that with titanium is most promising.[29]

Quercitin is separated from rutin by paper chromatography with 20:5:11 butanol-acetic-acid-water. The rutin spot is eluted with 1:1 methanol-0.2N HCl. Thereafter rutin is determined by reaction with diazotized *p*-aminobenzoic acid.[30]

Rutin also gives an orange-yellow in aqueous solution by 1:1 combination with uranyl ion.[31] Rutin is determined in pharmaceutical preparations by treating alcoholic solutions with titanium sulfate and reading the resulting color, which conforms to Beer's law, at 533 and 541 mμ.[32] Rutin and quercitin are determined in the same solution by a technic shown under quercitin, page 146.

Procedure—*Direct reading. Tablets.* Take a sample containing 0.05-0.5 gram of rutin and dissolve any coating with water. Add 20 ml. of acidified ethanol prepared by diluting 550 ml. of 95% ethanol and 50 ml. of glacial acetic acid to 1 liter with water. Break up the sample and heat at 70-80° for 10 minutes with stirring. Rinse the stirring rod with acidified alcohol and centrifuge the solution for 15 minutes. Decant and drain for 10 minutes. Rinse the mouth of the tube with acidified ethanol and repeat the extraction twice. Dilute the combined extracts to 250 ml. with acidified ethanol. Filter and discard the first part of the filtrate. Dilute to 0.01-0.03 gram of rutin per liter. Filter and discard the first 15-20 ml. of filtrate. Read at 338.5, 352.5, and 366.5 mμ against water.

$$\text{Determine } R_1 = A_{338.5}/A_{352.5}$$

$$\text{Determine } R_2 = A_{366.5}/A_{352.5}.$$

If $R_1 = 0.914 \pm 0.009$ and $R_2 = 0.842 \pm 0.013$ the extracted material is rutin without interferences.

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[28] J. Valentin and G. Wagner, *Pharm. Zentralhalle* 91, 291-310 (1952).

[29] P. Hagedorn and R. Neu, *Arch. Pharm.* 286, 486-90 (1953).

[30] J. Davidek and J. Fragner, *Československ. farm.* 6, 449-50 (1957).

[31] D. Gr. Constaninescu and V. Em. Calcandi, *Acad. rep. populare Romîne, Studii cercetări chim.* 4, No. 1-2, 99-109 (1956); T. Bross and U. Bilińska, *Acta Polon. Pharm.* 15, 39-43 (1958).

[32] Urszula Bilińska, *Acta Polon. Pharm.* 15, 123-8 (1958).

Then
$$a_{352.5} = \frac{A\ 352.5}{bc}$$
where

a = absorptivity
A = absorbance
b = cell length
c = concentration in grams per liter.

$$\text{Mg. rutin per tablet} = a\frac{352.5}{263.3} \times \text{average weight of tablet in mg.}$$

By alkali. Blood. Mix 5 ml. of sample with 15 ml. of 8% trichloroacetic acid. Filter or centrifuge. Mix 6 ml. of protein-free sample with 0.3 ml. of 50% sodium hydroxide solution and heat at 100° for 15 minutes. Read the yellow color for rutin.

Flower buds by aluminum ion. Extract the rutin with 99% methanol and evaporate the methanol solution to dryness. Extract quercitin from the dried residue by repeated treatment with diethyl ether. Take up the residue in 99% methanol and dilute to an appropriate concentration. Treat a 2-ml. aliquot with 3 ml. of 1.3% aluminum chloride solution and 5 ml. of 1% potassium acetate solution. Let stand for 2 hours at room temperature and read the absorption at 463 mμ.

$$\%\ \text{rutin} = (0.0612A_{463} - 0.00072) \pm 0.00049.$$

General by aluminum ion. To a 5-ml. aliquot of 3:2 alcohol containing up to 1 mg. rutin add 0.3 ml. of 5% sodium nitrite solution and 0.3 ml. of 10% aluminum trinitrate solution. Let stand for 3 minutes and add 0.5 ml. of 30% sodium hydroxide solution. Read the resulting red color at 413 mμ.

By p-*aminobenzoic acid.* Add 2 ml. of 0.2% sodium nitrite solution to 1 ml. of 0.5% *p*-aminobenzoic acid in 1:9 sulfuric acid. Stir until the yellow color disappears. Add the sample in methanol and make alkaline with 5 ml. of 10% sodium hydroxide solution. Dilute to 25 ml. with water and read at 420 mμ against a blank.

FLAVONOIDS

Flavonoids in tobacco are determined by an intense yellow developed with aluminum ion and potassium acetate.[33]

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[33] M. K. Mikhailov, *Compt. rend. acad. bulgare sci.* **11,** 493-6 (1958).

Procedure—Dry a pulverized sample of 0.13-0.17 gram at 50-55° for 12 hours. Wrap in filter paper and extract with anhydrous chloroform to remove resins and coloring matter. Reflux with 30 ml. of 90% ethanol for 10-15 minutes and decant the extract. Repeat with two similar extractions. Add the tobacco and decantates to a 100-ml. flask, cool, and dilute to volume. Filter.

To 5 ml. of filtrate add 3 ml. of 2.4% solution of aluminum chloride hexahydrate and 5 ml. of 9.8% potassium acetate solution. Read at 420 mμ after 5 minutes against 5 ml. of filtrate and 8 ml. of water. Rutin is an appropriate standard.

METHYL MANNOSIDES

Methyl mannosides, separated by an automatically controlled cellulose powder chromatographic column, give a satisfactory color reaction with anthrone.[34] Beer's law is followed up to 0.15 mg. per ml. for each of the following compounds: methyl α-*d*-mannopyranoside, methyl β-*d*-mannopyranoside, methyl α-*d*-mannofuranoside, methyl β-*d*-mannofuranoside, and *d*-mannose. The most satisfactory solvent is 10:3:3 butanol-pyridine-water. If the concentration of one component is small large errors are found when the component is preceded or followed by a large and relatively heavy band of material since the separation is incomplete under these conditions.

Procedure—The chromatographic column is shown in Figure 11. The column is completely automatic. When started it will begin collecting samples after a definite forerun and shut itself off after obtaining the last fraction. The forerun and fraction time are determined by timing the flow of 25 ml. from the column. The former is set on a GraLab timer, *a*, and the latter on the fraction collector timer, *b*, shown in the figure.

The heart of the automatic operation is a Fisher Electro Hosecock, *c*, modified as shown in the inset of Figure 11. A microswitch, *d*, was installed in the hosecock in such a way that when the hosecock closes the microswitch snaps open and locks it closed, at the same time switching off both the hosecock and the fraction cutter. The plug, *e*, is plugged into the timer and the fraction collector is plugged into a receptacle, *f*, installed in the hosecock. The microswitch, *g*, makes contact and activates the hosecock when a knob, *h*, attached to the collector turntable, just after the last tube to be collected, comes in contact with it. The hosecock can

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[34] Dwight F. Mowery, Jr., *Anal. Chem.* 29, 1451-3 (1957).

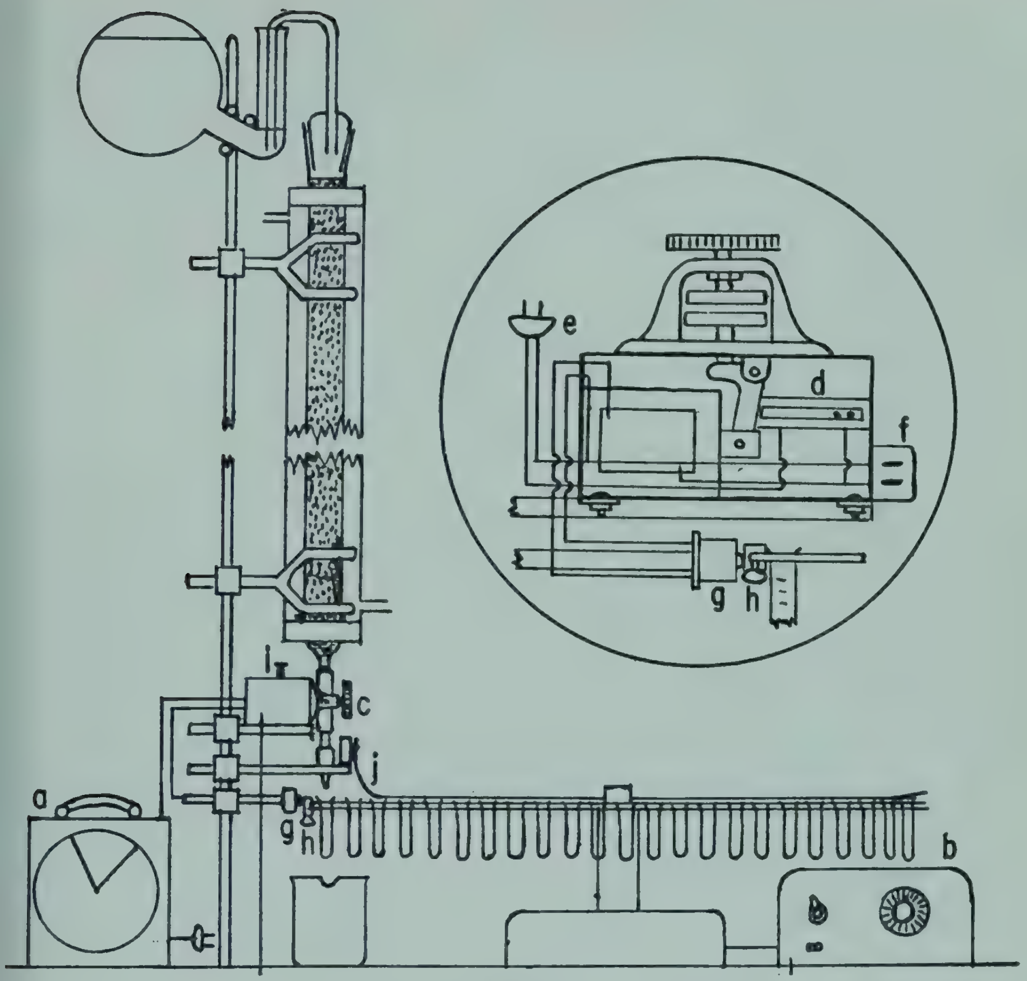

FIG. 11

Analytical chromatographic column equipped for automatic operation
Insert. Detail of Fisher Electro Hosecock modification

be opened by depressing a specially installed plunger, *i*, which pushes down the spring arm of the microswitch, *d*, inside, allowing the hosecock to open. Ninety 13-mm. tubes were used and a large rubber disk, *j*, is fastened to the top of the turntable so as to cover the tubes except when they are being filled, thus preventing dust contamination and evaporation. Pack the 1 × 30-inch column with 130 grams of cellulose powder as a slurry. Allow it to settle and apply a pressure of 20 pounds per square inch. To maintain a constant flow rate, use a constant solvent head and a 25° jacket, allowing the column to run unthrottled.

After the sample of approximately 20 mg. of the sample has been transferred to the column, chromatograph with a solvent containing 10 parts of butanol, 3 parts of pyridine, and 3 parts of water, collecting 5-ml. fractions. To a 1-ml. aliquot of each fraction, add 2 ml. of 0.2% anthrone solution in concentrated sulfuric acid. Mix and read after 1 hour at 620 mμ. Determine the areas under the various peaks of the absorbance plot with a planimeter and determine the percentage of a given isomer by multiplying the area under the peak by 100 and divide by the sum of areas under all peaks.

CONDURANGIN

Condurangin, $C_{35}H_{54}O_{14}(OCH_3)_2$, is determined by ferric chloride in an acidic medium.[35]

Procedure—Reflux a 1-gram finely powdered sample of condurangin bark with 50 ml. of 96% ethanol and restore the original weight with 96% ethanol. Filter, evaporate 25 ml. of the filtrate, and extract the residue with three 15-ml. portions and one 10-ml. portion of chloroform. Filter and evaporate the filtrate to 1-2 ml. Precipitate by adding 75 ml. of petroleum ether. After 2 hours, filter and dissolve the precipitate in 96% ethanol. Dilute to 50 ml. and evaporate a 2-ml. aliquot to dryness. Add 3 ml. of glacial acetic acid, 0.1 ml. of 5% ferric chloride solution, and 0.25 ml. of concentrated sulfuric acid. Place in the dark and read after 45 minutes at 610 mμ.

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[35] G. Zolss and L. Zechner, *Sci. Pharm.* 25, 163-72 (1957).

CHAPTER 11

ALDEHYDES

ONE CLASS of materials having multiple functional groups is the sugars. Those having aldehyde groups, such as fructose, appear in the sugar chapters. Others having multiple functional groups may appear elsewhere if determined by another group. For development of maleic aldehyde by *p*-aminophenol acid see page 259. The extinction coefficient of *n*-butyraldehyde as the 2,4-dinitrophenylhydrazone is 2.16 at 425 mμ and 0.898 at 530 mμ.

For separation of crotonaldehyde, 2-butene-1-al, from acetaldehyde by distillation see page 255. For reading in the ultraviolet along with acetaldehyde see page 254. In reading at 365 mμ the solutions at 0.1-12.8% follow Beer's law.[1] There is no serious interference by acetaldehyde, acetic acid, or acetone. For development by *p*-aminophenol acid see page 259.

The extinction coefficient of *n*-heptaldehyde as the 2,4-dinitrophenylhydrazone is 2.14 at 425 mμ and 0.843 at 530 mμ. Heptanoic aldehyde, enanthaldehyde, or oenenthaldehyde, in aqueous solution is determined nephelometrically by dinitrophenylhydrazine at 0.0015-0.015 mg. per ml.[2] The extinction coefficient of *n*-octylaldehyde as the 2,4-dinitrophenylhydrazone is 2.08 at 425 mμ and 0.868 at 530 mμ. The extinction coefficient of *n*-decylaldehyde, capric aldehyde as the 2,4-dinitrophenylhydrazone is 2.17 at 425 mμ and 0.873 at 530 mμ. It is also determined in aqueous solution nephelometrically at 0.0015-0.015 mg. per ml. by dinitrophenylhydrazine. The extinction coefficient of *n*-dodecylaldehyde as the 2,4-dinitrophenylhydrazone is 2.17 at 425 mμ and 0.900 at 530 mμ. The extinction coefficient of *n*-tetradecylaldehyde as the 2,4-dinitrophenylhydrazone is 2.08 at 425 mμ and 0.850 at 530 mμ.

Butanol extracts 5-(hydroxymethyl)furfural from glucose solution. Thereafter it is developed with benzidine in acetic acid and read at 400 mμ.[3] When read directly, 5,5′-(oxydimethylene)di-2-furfuraldehyde, bis(5-formyl-2-furfuryl ether), has a maximum in aqueous solution at 282.5 mμ.[4] Interferences by 5-hydroxymethyl-2-furfuraldehyde and furfural is serious; that by 2,5-bishydroxylfurane and by 5-hydroxymethyl-

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[1] K. P. Stolyarov and V. S. Ivanov, *Zhur. Anal. Khim.* 13, 246-9 (1958).

[2] M. Rothe and I. Voigt, *Ernährungsforschung* 2, 444-53 (1957).

[3] Y. Yoshihira and M. Nakamura, *Seizan Kenkyu* 10, 46-7 (1958).

[4] G. Mackinney and O. Temmer, *J. Am. Chem. Soc.* 70, 3586-90 (1948).

2-furoic acid is substantial. In ethanol the maximum is shifted to 281 $m\mu$.

Anisic aldehyde is estimated nephelometrically in aqueous solutio at 0.0015-0.015 mg. per ml. by dinitrophenylhydrazine.[6] For the determination of nitrofurantion by phenylhydrazine, follow the procedur for nitrofurazone, page 271.

Glyceraldehyde is also developed with *p*-hydroxybenzaldehyde, pag 44. Also *p*-hydroxybenzaldehyde is developed with titanium sulfate, pag 93. The maximum absorption is at 420 $m\mu$.

The methylene dioxyl group is $—OCH_2O—$. This is treated unde formaldehyde because it is cleaved by mineral acids to formaldehyd which is then determined.[7] The occurrence is as diverse as sesamin, alkaloids, anticarcinogens,[9] and other physiologically active agents o natural origin. Interferences also appear under formaldehyde.

Aldehyde-free methanol.[10] Heat 1 liter of anhydrous methanol wit 5 ml. of concentrated hydrochloric acid and 10 grams of 2,4-dinitriphenylhydrazine at 100° for 2 hours. Distil using a fractionating column an discard the first 5% and last 10% of the distillate.

Schiff's reagent. (Vol. III, pp. 251-2.) The optimum excess sulfu dioxide concentration is in the range of 2.8-4.8 millimoles of sulfu dioxide per 100 ml. of reagent.[11] After preparation of the reagent an calculation of the excess sulfur dioxide content, obtain the optimum rang by addition of sodium metabisulfite to raise the content or by bubblin air through the reagent to reduce the concentration.

For greater sensitivity, increase the dye content of the reagent t 300 mg. of dye per 100 ml. of reagent. Use vacuum filtration and reduc the contact time of activated carbon for decolorizing the reagent to 4 seconds. If the sulfur dioxide concentration is controlled it is no necessary to standardize the reagent each time unless there is a chang in the source of dye.

To 1500 ml. of water, add 4.5 grams of rosaniline hydrochloride. Swi to dissolve and add 9.6 grams of sodium metabisulfite. Mix, let stand fo 5-10 minutes, and add 40 ml. of 1:5 sulfuric acid. Mix, stopper, and le

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[5] J. H. Turner, P. A. Rebers, P. L. Barrick, and R. H. Cotton, *Anal. Chem.* 26 898-901 (1954).

[6] M. Rothe and I. Voigt, *Ernährungsforschung* 2, 444-53 (1957).

[7] Morton Beroza, *Anal. Chem.* 26, 1970-3 (1954).

[8] H. L. Haller, F. B. La Forge, and W. N. Sullivan, *J. Org. Chem.* 7, 185-8 (1942)

[9] J. L. Hartwell and A. W. Schrecker, *J. Am. Chem. Soc.* 73, 2909-16 (1951).

[10] Horst Böhme and Otto Winkler, *Z. anal. Chem.* 142, 1-5 (1954).

[11] D. E. Kramm and C. L. Kolb, *Anal. Chem.* 27, 1076-9 (1955).

stand overnight. Add 3 grams of activated carbon to decolorize the reagent, quickly mix by swirling, let stand for 45 seconds, and filter by vacuum through 2 sheets of filter paper as rapidly as possible, certainly in not more than 3 minutes. To a 10-ml. aliquot add 20 ml. of water and 5 ml. of 0.5% starch solution. Titrate the free sulfur dioxide to a starch end point with standard iodine solution. Calculate free sulfur dioxide as follows:

$$5 \times \text{ml. of iodine} \times \text{normality} = \text{millimoles of sulfur dioxide per 100 ml. of reagent.}$$

Adjust the sulfur dioxide range to the optimum 2.8-4.8 millimoles per 100 ml. of reagent if necessary, by adding sodium metabisulfite to raise the range, or by bubbling air through the reagent to lower the concentration.

Aldehydes in General

Aldehydes can be separated from ketones by chromatography.[12] As applied to acetone-furfural, methylethyl ketone-acetaldehyde, and acetone-acetaldehyde the mixture is sorbed on Amberlite IRA-400 in bisulfite form. The latter is formed by treating the resin with a solution of sodium bisulfite and washed free of sulfite ion. Appropriate resin particles are 0.12-0.30 mm. and an appropriate column 9.8 × 550 mm. The ketones are eluted with 300 ml. of water at 2 ml. per minute. Thereafter the aldehydes are eluted at the same rate with 6% sodium chloride solution.

Mixed saturated aliphatic carbonyl compounds can be converted to their 2,4-dinitrophenylhydrazones and separated by partition between petroleum ether and nitromethane on a silica gel column.[13] The silica gel can be replaced by diatomaceous earth.[14] On separation and identification the fractions are read at 425 mμ and 530 mμ.

In approximate determination of mixed aldehydes a reading on the mixed 2,4-dinitrophenylhydrazones is applicable. Thus 8 aldehydes from acetaldehyde to *n*-tetradecyl aldehyde have an average extinction coefficient of 2.13 at 425 mμ and 0.871 at 530 mμ. Table 16. The corresponding average values for 8 ketones are 1.87 at 425 mμ and 0.888 at 530 mμ. A yellow to orange color forms when aldehydes are treated with

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[12] G. Gabrielson and O. Samuelson, *Svensk. Kem. Tid.* **64**, 150-6 (1952).

[13] P. J. G. Kramer and H. Duin, *Rec. trav. chim.* **73**, 63-7 (1954).

[14] Kenneth J. Monty, *Anal. Chem.* **30**, 1350-2 (1958).

TABLE 16. MOLAR EXTINCTION COEFFICIENTS FOR SOME 2,4-DINITROPHENYLHYDRAZONES

	Extinction coefficient × 10^{-4} [a]	
Parent compound	425 mμ	530 mμ
Acetaldehyde	2.06	0.863
n-Propionaldehyde	2.08	0.813
n-Butyraldehyde	2.16	0.898
n-Heptaldehyde	2.14	0.843
n-Octyl aldehyde	2.08	0.868
n-Decyl aldehyde	2.17	0.873
n-Dodecyl aldehyde	2.17	0.900
n-Tetradecyl aldehyde	2.08	0.850
Aldehyde av.	2.13	0.871
Acetone	1.79	0.850
Methyl ethyl ketone	1.79	
Methyl *n*-butyl ketone	1.86	0.870
Methyl *n*-hexyl ketone	1.91	0.910
Methyl *n*-nonyl ketone	1.90	0.873
Diethyl ketone	1.81	0.865
Di-*n*-propyl ketone	1.89	0.923
Di-*n*-butyl ketone	1.85	0.913
Methyl isopropyl ketone	1.88	0.863
Ketone av.	1.87	0.888

[a] Absorbance per mole per liter per 1-cm. light path, as determined at 25°C.

phloroglucinol.[15] Aliphatic aldehydes and ketones in general react with vanillin in alkaline solution.[16] The reaction hardly occurs at all with aromatic compounds.

Procedure—*As dinitrophenylhydrazones.* With stirring, add 6 ml. of nitromethane to 6 grams of Hyflo Super-Cel. Suspend this mixture in hexane saturated with nitromethane to produce a slurry. Pack a 9 × 250 mm. column, using a tamping rod for uniform packing. Wash the column with 15-20 ml. of hexane saturated with nitromethane. Introduce the sample in nitromethane and adjust the flow rate to 0.8-1.2 ml. per minute at 25°. Collect 1-ml. fractions. Evaporate the fractions to dryness since the nitromethane will interfere. Dissolve each in 1 ml. of benzene and add 0.5 ml. of 4% potassium hydroxide solution in absolute ethanol. Read each fraction at 425 and 530 mμ over a period of time and obtain the zero-time absorbances at each wave length by graphic extrapolation of the plot absorbance against time after the addition of the potassium hydroxide. From the average values for the molar extinction coefficients at the two wave lengths, aldehydes, and ketones are calculated as follows:

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[15] J. Petranek and M. Vecera, *Chem. Listy* 51, 1686-9 (1957).

[16] V. E. Levine and M. Taterka, *Anal. Chim. Acta* 15, 237-45 (1956).

$$\text{conc.}_{\text{ald.}} = A_{425} - (2.10 \times A_{530})$$

$$\text{conc.}_{\text{ket.}} = \frac{(2.45 \times A_{530}) - A_{425}}{1.02}.$$

By phloroglucinol. Dissolve a sample in glacial acetic acid which has been purified by distillation from 0.5% phloroglucinol solution in 1:19 sulfuric acid. Treat a 5-ml. sample with 5 ml. of 1:4 sulfuric acid in glacial acetic acid. Stir and cool to 18-20°. Add 1 ml. of 1% phloroglucinol solution in glacial acetic acid and read after 10-25 minutes against 1:9 sulfuric acid in acetic acid.

By vanillin. To 1-2 ml. of sample solution add 1 ml. of 1% vanillin solution and 2 ml. of 20% sodium hydroxide solution. Heat at 100° for 10 minutes and cool. Read at 508 mμ.

FORMALDEHYDE

The familiar reaction of Schiff's reagent with aldehydes is applicable to determination of formaldehyde (Vol. III, p. 258), with possible limitations depending on the accompanying substances. Thus, mixtures of formaldehyde, acetaldehyde, and peroxides are products of partial oxidation of low-molecular-weight hydrocarbons. After reduction of the peroxides the formaldehyde is determined with Schiff's reagent.[17] If the reaction solution is not strongly acid, acetaldehyde will interfere.

Since hydrochloric acid interferes with the reaction of formaldehyde and Schiff's reagent, sulfuric acid is used on the development of the color, although hydrochloric acid may be used as a solubilizing and hydrolyzing agent for the sample if necessary. At low formaldehyde concentrations, glacial acetic acid is substituted for water in the reaction mixture to increase the sensitivity of the reagent.[18]

One determination of compounds which are not formaldehyde but form it is that of the methylenedioxyl group which is based on its hydrolytic cleavage by mineral acids to formaldehyde which is then determined by chromotropic acid.[19] An absorbance equivalent to 0.75 to 1.08 moles of formaldehyde is obtained per mole of methylenedioxyl group.

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[17] C. N. Satterfield, R. E. Wilson, R. M. LeClair, and R. C. Reid, *Anal. Chem.* 26, 1792-7 (1954).

[18] D. E. Kramm and C. L. Kolb, *Ibid.* 27, 1076-9 (1955).

Another application of this technic of determination of the methylenedioxyl group is to the determination of piperine in pepper.[20] With samples which form a precipitate in sulfuric acid during the reaction, the ratio of solvent to sulfuric acid should be increased. As the acid concentration increases above 2:1, the wave length of the maximum absorbance shifts and there is an increase in absorptivity. Dilution with sulfuric acid instead of water makes the procedure simpler and faster. Diethoxymethane is recommended as the standard.

Methylene groups attached to nitrogen which liberate formaldehyde interfere. Examples of these compounds are *N*-methylene-glycinonitrile, *N,N′*-methylenebisacrylamide, and hexamethylenetetramine. No formaldehyde is produced by the —N=CH—N= group such as in histidine dihydrochloride or uric acid, by the =N—CO—N= group in urea, by the =N—CH_2CH_2—N= group in sparteine sulfate, the =N—CNH—N= group in guanidine nitrate, or the formyl(2,6-formoxylidide) groups. Methylene groups attached to sulfur such as in *s*-trithiane liberate formaldehyde. Primary carbinol groups interfere and are eliminated by oxidation to the acid. Aromatic or aromatic-type carboxylic acids are quantitatively decarboxylated by heating them in quinoline in the presence of a catalyst. Esterification of the carbinol group causes less formaldehyde to be formed. All sugars which produce 5-hydroxymethylfurfural interfere. 2,4-Dichlorophenoxyacetic acid and 2,4-dichlorophenol do not interfere. Phenol, resorcinol, and phloroglucinol compete with formaldehyde for the reagent, but this interference can partially be overcome by increasing the concentration of reagent.

The results on pepper are not only piperine but include chavicine and other isomers of piperine. This is only a minor inaccuracy as the amounts present are small and they also contribute to the pungency of the spice.

The phenylhydrazone of formaldehyde, oxidized with potassium ferrocyanide forms an orange color when treated with hydrochloric acid.[21] The color development takes an appreciable time. It is either read directly in aqueous solution or after extraction with butanol. Yet another alternative is to treat with excess sodium hydroxide[22] after the color is developed. Increasing the concentration of reagent in phenylhydrazine and potassium ferrocyanide and reducing the final volume of solution improves the intensity and stability of the color.

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[19] Morton Beroza, *Ibid.* **26**, 1970-3 (1954).

[20] Leonard A. Lee, *Ibid.* **28**, 1621-3 (1956).

[21] A. D. Marenzi and E. S. Braegger, *Publs. inst. invest. Microquim., Univ. nacl. litoral.* (*Rosario, Arg.*) **17**, 158-69 (1953); Daniel K. Owens, *Sewage and Ind. Wastes* **27**, 939-40 (1955); R. C. Hirt, W. R. Doughman, R. G. Schmitt, and S. T. Moore, *J. Agr. Food Chem.* **3**, 1044-6 (1955).

A methanol solution of 2,4-dinitrophenylhydrazine is a reagent for both formaldehyde and vanillin.[23] Fluoroglycine also produces a red color when reacted with formaldehyde.[24] The presence of a 10-fold excess of methanol and acetone and the presence of 5-10 times the amount of formic acid has no effect on the method. Beer's law is followed.

At pH 5.5-6.5 acetylacetone and ammonium salts react quantitatively with formaldehyde producing a yellow color due to the synthesis of diacetyldihydrolutidine. The concentration of reagents is limited within the range of 0.1-1 M ammonium hydroxide and 0.001-0.1 M acetylacetone.[25] Sulfite interferes.

Samples—*Cellulose acetate formal.*[26] To a sample of 1-1.5 grams add 10 ml. of 3:1 hydrochloric acid, stopper, and swirl vigorously. During 2 hours occasionally swirl the flask vigorously and then let stand overnight at room temperature. Stopper and heat for 2 hours at 35-50°. Cool to room temperature, add 90 ml. of 1:1.6 sulfuric acid, and mix. Add an aliquot of 10 ml. or less to 20 ml. of water. Use of a larger aliquot causes interference by hydrochloric acid. Develop by Schiff's reagent.

Methylenedioxyl compounds. Select a sample to contain about 0.05 mg. of the radical. Place 1 ml. of sample in acetone or other readily volatile solvent in a tube connected by a ground-glass joint to an adapter which is in turn connected to a water pump and manometer. Evaporate the solvent under reduced pressure while swirling in water at room temperature. Develop in this tube by chromotropic acid.

Pepper. Free and combined formaldehyde.[27] Mix a 0.5-gram sample of ground pepper in 95% ethanol. Dilute with ethanol to about 200 ml. and boil for 3 hours with occasional swirling. Cool, dilute to 250 ml. with 95% ethanol, and filter or centrifuge a small aliquot of the extract. Develop by the chromotropic acid method for free and combined formaldehyde.

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[22] N. Zurlo and A. M. Griffini, *Med. lavoro* **45,** 692-4 (1954); T. Fukuyama, T. Sato, A. Watanabe, and H. Kimura, *Bull. Inst. Public Health* (*Tokyo*) **5,** No. 3 1-6 (1956).

[23] R. Kalvoda and J. Zýka, *Farmácia* **23,** 197-9 (1954).

[24] C. Almassy and E. Zichy, *Magyar Kem. Folyoirat* **62,** 106-9 (1956).

[25] T. Nash, *Nature* **170,** 976 (1952); T. Nash, *Biochem. J.* **55,** 416-21 (1953).

[26] D. E. Kramm and C. L. Kolb, *Anal. Chem.* **27,** 1076-9 (1955).

[27] Leonard A. Lee, *Ibid.* **28,** 1621-3 (1956).

Formaldehyde-tanned collagen.[28] Reflux formaldehyde-tanned collagen with 1:2 sulfuric acid for 1 hour to hydrolyze. Dilute so that the final solution will contain approximately 0.09 mg. of formaldehyde per ml. Develop with chromotropic acid.

Air. Pass air through 50 ml. of water at the rate of 1 liter per minute until 0.005-0.01 mg. is collected. Develop with phenylhydrazine and potassium ferrocyanide[29] or chromotropic acid.[30]

Procedure—*By Schiff's reagent.*[31] To a sample containing up to 0.4 mg. of formaldehyde, diluted to 30 ml. with water, add 20 ml. of reagent. Mix and let stand for 2 hours. In pipetting the reagent, do not apply suction which would result in loss of sulfur dioxide. Read at 550 mμ with a photometer adjusted for 100% transmittance against a blank containing 20 ml. of reagent and the acids used in preparation of the sample.

By chromotropic acid.[32] The solid sample should contain about 0.05 mg. of formaldehyde or equivalent. Add 1 ml. of 10% chromotropic acid solution and 5 ml. of concentrated sulfuric acid with continuous shaking. Swirl vigorously and heat at 100° for 30 minutes. Cool under cold water and dilute to 50 ml. with 1:1 sulfuric acid. Mix by inverting. Cool to room temperature and read at 570 mμ against a reagent blank. At the same time determine the absorbance developed by 1 ml. of solution containing a known amount of trioxane or formaldehyde with the following modifications. Do not evaporate the solvent. Add 100 mg. of solid chromotropic acid in place of the chromotropic acid solution. If the absorbance of the unknown is more than that produced by 0.037 mg. of trioxane or formaldehyde take a smaller quantity of the sample.

If the sample is volatile or insoluble in a volatile solvent, dissolve it in water and substitute 100 mg. of solid chromotropic acid for the reagent solution.

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[28] S. M. Bose, K. T. Joseph, and B. M. Das, *Tanner (India)* **4**, No. 3, 23; No. 4, 16-20 (1956).

[29] N. Zurlo and A. M. Griffini, *Med. Lovaro* **45**, 692-4 (1954).

[30] W. E. MacDonald, Jr., *Am. Ind. Hyg. Assoc. Quart.* **15**, 216-9 (1954).

[31] D. E. Kramm and C. L. Kolb, *Anal. Chem.* **27**, 1076-9 (1955).

[32] Morton Beroza, *Ibid.* **26**, 1970-3 (1954).

Free and combined formaldehyde.[33] Place a 1-ml. aliquot in each of two flasks and prepare a third flask containing 1 ml. of 95% ethanol. To the first and third flask add 0.5 ml. of 25% chromotropic acid solution and add 10 ml. of concentrated sulfuric acid to all three flasks. Swirl to mix and heat at 100° for 30 minutes, stoppering loosely after 1-2 minutes. Cool to room temperature, dilute to 25 ml. with concentrated sulfuric acid, and mix. Read the first two flasks containing samples at 580 mμ, the first flask containing chromotropic acid against the third blank flask, and the second flask containing no reagent against sulfuric acid.

If the sample was piperine then 0.66 = the absorbance of 1 micromole in 25 ml. as formaldehyde.

$$\%\ \text{piperine} = 10.81\left(\frac{A - B}{\text{sample wt. in grams}}\right)$$

where A = absorbance of sample
B = absorbance of blank.

If the second flask contains no colored salt of the hydrolytic products of piperine with sulfuric acid and therefore is omitted, calculate piperine as follows:

$$\%\ \text{piperine} = 10.81\left(\frac{0.9A}{\text{sample wt. in grams}}\right).$$

By phenylhydrazine and potassium ferrocyanide.[34] Dilute a sample containing 0.005-0.01 mg. of formaldehyde to 10 ml. with water. Add 2 ml. of isopropanol and 1.5 ml. of 7.5% phenylhydrazine solution. After 10 minutes, add 0.5 ml. of 5% potassium ferrocyanide solution. Let stand for 5 minutes. Add 2 ml. of 10% sodium hydroxide solution. Read at 530 mμ after 30 minutes.

Alternatively[35] after "Let stand for 5 minutes," add 4 ml. of concentrated hydrochloric acid and 15 ml. of butanol. Shake, separate the upper layer, and read at 520 mμ.

Lard. Dissolve a 15-gram sample in 30 ml. of isooctane and extract four times with 3-ml. portions of water. Combine the first two and the last two extracts for separate determination. Treat 6 ml. of the aqueous extract of the sample containing a minimum of 0.0003 mg. of formaldehyde with 1 ml. of 1% phenylhydrazine hydrochloride solution and let stand for 10 minutes. Add 0.5 ml. of potassium ferricyanide solution followed immediately by 2 ml. of concentrated hydrochloric acid and dilute to 10 ml. Read after 10 minutes at 520 mμ and subtract a blank.

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[33] Leonard A. Lee, *Ibid.* 28, 1621-2 (1956).

[34] N. Zurlo and A. M. Griffini, *Med. lavoro* 45, 692-4 (1954).

[35] Daniel K. Owens, *Sewage and Ind. Wastes* 27, 939-40 (1955).

By 2,4-dinitrophenylhydrazine.[36] To a 2-ml. sample in methanol, add 1 ml. of a saturated 2,4-dinitrophenylhydrazine solution in methanol, and 2 drops of concentrated hydrochloric acid. Heat at 50-60° for 1 hour and cool. Add 5 ml of 10% potassium hydroxide solution in methanol and 14 ml. of methanol. Read at 525 mμ.

By acetylacetone and ammonium acetate.[37] Prepare the reagent by dissolving 150 grams of ammonium acetate, 3 ml. of glacial acetic acid, and 2 ml. of redistilled colorless acetylacetone in water and diluting to 1 liter. Mix the reagent with an equal volume of solution containing not more than 0.008 mg. of formaldehyde per ml. Read at 412 mμ after 5 hours if the solution is kept at 20°, after 40 minutes at 37°, or after 5 minutes at 58°.

By fluoroglycine. To a solution containing 1-4 mg. of formaldehyde, add 3 ml. of a fresh 1% fluorogylcine solution and 2 ml. of 10% sodium hydroxide solution. Dilute to 50 ml. and read at 463 mμ after 1.5-2 minutes and before 3 minutes.

Acetaldehyde

Acetaldehyde in concentrations of 0.005-0.01% in acetic acid is determined by ultraviolet spectrophotometry at 281 mμ with an accuracy of 0.2%.[38] Acetaldehyde and crotonaldehyde are determined in the presence of each other by their absorption peaks in purified vinyl acetate read against solvent.[39] Since the absorption peak for acetaldehyde is at 287 mμ and that for crotonaldehyde at 325 mμ follow Beer's law for concentrations in the range of 0.01-0.5%, the concentration of the two aldehydes is calculated by solving simultaneous equations.

Acetaldehyde can also be determined in acetic acid in the presence of crotonaldehyde by heating with sodium bisulfite and distilling off the acetaldehyde in the presence of sodium bicarbonate.[40] 2,4-Dinitrophenylhydrazine in methanol is another reagent for acetaldehyde.[41] The extinction coefficient as the 2,4-dinitrophenylhydrazone is 2.06 at 425 mμ and 0.063 at 530 mμ. For those of other ketones and aldehydes see Table 16.

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[36] R. Kalvoda and J. Zýka, *Farmácia* **23**, 197-9 (1954).
[37] T. Nash, *Biochem. J.* (*London*) **55**, 416-21 (1953).
[38] C. Capitani and E. Milani, *Chim. e ind.* (*Milan*) **38**, 671-3 (1956).
[39] C. Capitani and E. Milani, *Ibid.* **37**, 177-82 (1955).
[40] Eero Sjoström, *Acta Chem. Scand.* **7**, 1392-4 (1953).

In the determination of acetaldehyde by Schiff's reagent (Vol. III, p. 261) five different compounds have been identified spectrophotometrically in the reaction mixture.[42] With a small amount of acetaldehyde a complex with one mole of acetaldehyde for every mole of reagent is formed, the compound having a peak absorption at 545 mμ. With excess acetaldehyde the complex contains two moles of acetaldehyde per mole of reagent with a peak at 555 mμ. After standing this compound shows the presence of additional compounds with peaks at 525 mμ, 575 mμ, and 615 mμ. The first compound results from the interaction of two moles of acetaldehyde and one mole of fuchsin from the Schiff's reagent. The other compounds are formed when the first compound loses water. Acetaldehyde is also developed with *p*-hydroxybenzaldehyde, page 44.

Separation of Acetaldehyde from Crotonaldehyde.[43]—To 10-100 ml. of solution containing less than 0.5 gram of acetaldehyde and crotonaldehyde add 25 ml. of 10% sodium bisulfite solution and let stand overnight at 70-80°. Add 30 ml. of 8.4% sodium bicarbonate solution and distil approximately 25 ml. containing the liberated acetaldehyde with the tube dipping below the surface of the receiver. Collect in 10 ml. of 2% sodium bisulfite solution immersed in ice. After 30 minutes determine 0.002-0.0002 mg. of acetaldehyde per ml. The crotonaldehyde is in the residue.

Procedure—*By 2,4-dinitrophenylhydrazine.*[44] To 1 ml. of 2,4-dinitrophenylhydrazine reagent prepared by suspending 50 mg. of reagent in 30 ml. of carbonyl-free methanol add 1 ml. of sample. Let the mixture stand for 30 minutes and add 5 ml. of a mixture prepared by mixing 4 volumes of pyridine which has been kept over potassium hydroxide, 1 ml. of water, and 1 ml. of potassium hydroxide solution prepared by dissolving 10 grams of potassium hydroxide in 20 ml. of water and diluting to 10 ml. with carbonyl-free methanol. Read at 550 mμ after 10 minutes, against a blank consisting of 1 ml. of the 2,4-dinitrophenylhydrazine reagent and 1 ml. of water similarly treated.

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[41] H. Böhme and O. Winkler, *Z. anal. Chem.* **142,** 1-5 (1954).

[42] S. N. Bhattacharya, *Anal. Chim. Acta* **11,** 463-6 (1954).

[43] Eero Sjoström, *Acta Chem. Scand.* **7,** 1392-4 (1953).

[44] H. Böhme and O. Winkler, *Z. anal. Chem.* **142,** 1-5 (1954).

METALDEHYDE

Metaldehyde, a tetramer of acetaldehyde having an 8-membered ring, is used for control of slugs and snails. Since it is poisonous to warm-blooded animals it is necessarily determined on plant materials. An indirect method is based on the conversion of metaldehyde to acetaldehyde by steam distillation in the presence of sulfuric acid. The acetaldehyde is then determined by *p*-phenylphenol in the presence of sulfuric acid containing a trace amount of cupric ion.[45]

The method is extremely sensitive and can be affected by chromic acid used in cleaning glassware, lubricating grease, perspiration from the hands, and breath used in blowing the reagent in from a pipet. Chlorophyll does not interfere. Incomplete removal of chloroform, the extracting solvent, inhibits color development. Beer's law is followed for 0.0005-0.008 mg. of metaldehyde at 590 mμ. The color is stable for at least 2 hours.

Procedure—*Plant material.* Extract a weighed, chopped sample with a measured volume of chloroform for at least 1 hour, using at least 200 ml. of solvent for each 100 grams of green sample. Filter through dry, acetone-washed cotton. To remove free acetaldehyde, add 3 ml. of cold, freshly prepared 2% sodium bisulfite solution for each 50 ml. of chloroform extract. Shake for 1 minute. Separate and extract the chloroform solution four times with 10-ml. portions of water for each 50 ml. of chloroform. Filter through dry acetone-washed cotton.

Prepare a steam-distillation apparatus, placing the flask and receiving tube in ice baths. Add 3 ml. of 2% sodium bisulfite solution to the receiving tube. Put 10 ml. of 1:250 sulfuric acid, an aliquot of the sample, and a quartz pebble in the distillation flask. Distil slowly as acetaldehyde until 3-4 ml. of liquid remains in the distillation flask.

Remove the flask and rinse down the inside of the condenser and the inside and outside of the delivery tube with water. Decant the aqueous layer from the receiving tube and dilute to 50 ml. with water. To remove any remaining chloroform, shake with 5 ml. of *n*-hexane.

Add a 1-ml. aliquot to 8 ml. of a cupric sulfate-sulfuric acid reagent immersed in ice, prepared by mixing 5 ml. of 5% cupric sulfate pentahydrate solution with 500 ml. of concentrated sulfuric acid. Mix and add 4 drops of *p*-phenylphenol reagent prepared by dissolving 1 gram of *p*-phenylphenol in 25 ml. of hot 8% sodium hydroxide solution and adding 75 ml. of water before cooling. Gently agitate to disperse the reagent and let stand at 20-30° for 1 hour. Heat the sample containing the reagent at

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[45] P. A. Giang and F. F. Smith, *J. Agr. Food Chem.* **4**, 623-5 (1956).

100° for 90 seconds, cool to room temperature, and read the violet color against a reagent blank at 590 mμ.

PROPIONALDEHYDE

In concentrated sulfuric acid, propionaldehyde reacts with ninhydrin to produce a deep red-blue color which can be read at 595 mμ. The reaction is specific for propionaldehyde thus permitting its determination in the presence of other aldehydes.[46] The color develops at a faster rate at higher temperatures, but a temperature of 25° is chosen since the color is not stable over 50°. The color should be read within 15 minutes after addition of sulfuric acid since it is not stable beyond that time. The reagent is prepared in sodium bisulfite solution to inhibit its oxidation and increase its stability. Beer's law is followed.

The following aldehydes produce no color with ninhydrin: formaldehyde, paraformaldehyde, acetaldehyde, paraldehyde, isobutyraldehyde, crotonaldehyde, 2-furaldehyde, benzaldehyde, vanillin salicylaldehyde, and acrolein. Although formaldehyde does not react with the reagent, it must be removed with glycine since it inhibits the reaction between propionaldehyde and ninhydrin. *n*-Butylaldehyde gives a red color with the reagent which absorbs at 470 mμ but if present in equal or smaller amounts than propionaldehyde, *n*-butylaldehyde will not interfere. 2-Butanone and small amounts of acetone do not interfere. One mg. of acetone gives a color corresponding to 0.004 mg. of propionaldehyde. The amino acids, glycine, *dl*-valine, *dl*-lysine, *l*-proline, *l*-hydroxyproline, *l*-tyrosine, and *l*-arginine, do not react with ninhydrin under the conditions of the method.

The extinction coefficient as the 2,4-dinitrophenylhydrazone is 2.08 at 425 mμ and 0.813 at 530 mμ. For those of other aldehydes and ketones see Table 16. Propionaldehyde is also developed with *p*-hydroxybenzaldehyde, page 44.

Samples—*Air*. Prepare an aeration train containing 20 ml. of 2% glycine solution in saturated sodium bicarbonate in the first tube, 20 ml. of 5% sodium bisulfite solution in the second tube and third tubes. Pass the air containing the propionaldehyde through the train at the rate of 0.2 liter per minute for a definite interval. Combine the second and third tubes and dilute with 5% sodium bisulfite solution so that a 0.5-ml. aliquot contains up to 0.03 mg. of propionaldehyde. Develop with ninhydrin.

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[46] L. R. Jones and J. A. Riddick, *Anal. Chem.* **26**, 1035-8 (1954).

Aldehydes, formaldehyde absent. Dilute a sample to a known volume with 5% sodium bisulfite solution. Dilute an aliquot to a known volume with 5% sodium bisulfite so that 0.5 ml. will contain about 0.03 mg. of propionaldehyde.

Mixed aldehydes, formaldehyde present. Prepare an aeration train containing four tubes. To the first add 20 ml. of a saturated sodium bicarbonate solution, to the second 20 ml. of 5% glycine solution in saturated sodium bicarbonate solution, to the third 20 ml. of 5% sodium bisulfite solution, and to the fourth 15 ml. of 5% sodium bisulfite solution. Add 1 ml. of sample containing 1-3 mg. of propionaldehyde in sodium bisulfite solution to the first tube. Connect all tubes and adjust the air flow to a rate of 0.2 liter per minute as measured by a flowmeter. Aerate for 3 hours and quantitatively combine the third and fourth tubes. Dilute to 50 ml. with water and develop propionaldehyde with ninhydrin.

Procedure—*By ninhydrin.* Dilute an aliquot containing 0.005-0.03 mg. of propionaldehyde in 5% sodium bisulfite solution to 50 ml. with water. To a 0.5 ml. aliquot add 0.1 ml. of water and cautiously add 4 ml. of concentrated sulfuric acid. Mix and cool to 25° under water. Add 0.2 ml. of 3% ninhydrin solution in 5% sodium bisulfite solution and mix. Stopper and let stand for 1 hour at 25°. Dilute to 12:5 ml. with concentrated sulfuric acid, mix, and read at 595 mμ against a reagent blank.

MALONALDEHYDE

Oxidized lipids form a red compound with 2-thiobarbituric acid which is a measure of oxidative rancidity in fatty foods. The reactive compound in the fats is malonaldehyde.[47] It is believed formed by hydrolysis of 1,1,3,3-tetraethoxypropane. This latter is therefore a more appropriate standard after hydrolysis than malonaldehyde itself.[48]

Procedure—*Fish meal.* Vacuum-dry at 100° and grind. Mix a 0.22-0.26 gram sample with 4 ml. of water, 5 ml. of a 30:10 mixture of pyridine and 1:1 hydrochloric acid, 10 ml. of 20% trichloroacetic acid solution, and 6 ml. of 2% aqueous thiobarbituric acid. Reflux over boiling water for 30 minutes and add through the condenser 75 ml. of a reagent containing 650 ml. of 1:10 hydrochloric acid, 50 ml. of 20% trichloroacetic acid, and 50 ml. of a mixture of equal parts of pyridine and 1:1 hydrochloric acid. Continue to reflux for 10 minutes. Cool and centrifuge an aliquot. Extract 15 ml. with 10 ml. of hexane for 0.5 minute. Centrifuge to give a clear aqueous layer; otherwise extract with another 10 ml. of hexane. Read at 535 mμ subtracting a reagent blank.

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[47] S. Patton and G. W. Kurtz, *J. Dairy Sci.* 34, 669-74 (1951); W. G. Jennings, W. L. Dunkley, and H. G. Rieber, *Food Research* 20, 13-22 (1955).

[48] T. C. Yu and R. O. Sinnhuber, *Food Technol.* 11, 104-8 (1957); R. O. Sinnhuber and T. C. Yu, *Ibid.* 12, 9-12 (1958).

Fresh fish. Grind and mix an appropriate sample starting at mixing ". . . with 4 ml. of water, 5 ml. . . ."

Fish fillet and canned fish. Disintegrate in a blender and use an appropriate sample starting at mixing ". . . with 4 ml. of water, 5 ml. . . ."

FUMARALDEHYDE

Fumaraldehyde and other unsaturated 2,3-aldehydes produce a yellowish-green color when treated with *p*-aminophenol in trichloroacetic acid.[49] The concentration of the trichloroacetic acid must be known since the color increases linearly with the trichloroacetic acid concentration. The color is stable for 2 hours in 6% tricholoracetic acid solution.

The same color development at the same intensity is given by maleic aldehyde, and at varying intensities by cinnamic aldehyde, furfural, and crotonaldehyde. Saturated aldehydes, acetone, alcohols, mesityl oxide, and 2,3-unsaturated ketones do not react. Acrolein gives a color which fades rapidly. Cysteine and glutathione form compounds with equimolecular amounts of the reagent.

Procedure—To 1-4 ml. of aqueous sample solution, which may contain methanol or acetone, add 0.5-3.5 ml. of 40% trichloroacetic acid solution. Dilute to 8 ml. with water. Add 2 ml. of 3% colorless solution of *p*-aminophenol in 6% aqueous trichloroacetic acid solution. Read at 434 mμ. If the final solution contains 6% of trichloroacetic acid the color, which develops at once, is stable for at least 2 hours.

ISOBUTYRALDEHYDE

The determination of isobutyraldehyde in *n*-butyraldehyde is accomplished by reducing both aldehydes to alcohols with sodium borohydride and treating with concentrated sulfuric acid and salicylaldehyde in ethanol. Under these conditions the resulting isobutanol gives a color 100-150 times deeper than that produced with an equal quantity of butanol.[50] Factors which influence color development include rate of addition of reagents, mixing, temperature, and total amounts of ethanol and water. The heat of reaction is controlled so that the duration and temperature of heating can be reproduced.

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[49] F. L. Breusch and E. Ulusoy, *Hoppe-Seyler's Z. physiol. Chem.* **291**, 64-7 (1952).

Since other aldehydes also react with salicylaldehyde and sulfuric acid, they must be left behind by flash distillation. To prevent oxidation of the distillate, a large volume of water is used in the distillation. The reducing reagent is stable up to one day. The length of time allowed for the aldehydes to stand with the reducing solution is not critical.

Procedure—*Isobutyraldehyde in* n-*butyraldehyde.* Dilute a 1-ml. sample in ethanol to 50 ml. with water and flash-distil into an ice-cooled flask for 3-4 minutes beyond the point when no drops are visible in the condenser. Dilute the distillate to 100 ml. with water and take two 10-ml. aliquots. Determine the total aldehyde concentration in one aliquot.

To another aliquot, add 10 ml. of 0.15% sodium borohydride solution, stopper, and let stand for at least 10 minutes. Excess must be present at the end of the reaction. Dilute to 50 ml. with water. Calculate the volume of reduced aldehyde solution required to be equivalent to exactly 15 mg. of total butanol from the total aldehyde concentration found, and dilute this calculated volume to 50 ml. with absolute aldehyde-free ethanol. To estimate approximately 4% of isobutyraldehyde in *n*-butyraldehyde, the total butanol concentration of 0.03 gram per 100 ml. is chosen.

Carefully layer 3 ml. of 0.16% salicylaldehyde solution in aldehyde-free 95% ethanol on 10 ml. of concentrated sulfuric acid in a 7 × 1-inch test tube. Similarly add a layer of a 3-ml. aliquot of the sample. Immediately immerse both in a solid carbon dioxide-ethanol mixture at approximately −78° and swirl vigorously for 15 seconds.

Remove and again mix thoroughly by swirling. The contents of the tube should be slightly below or at room temperature. Heat for 80° for 30 minutes, noting the time, and cool under water. Read at 550 mμ 10 minutes after removed from the water. Then read the proportion of isobutyraldehyde and *n*-butyraldehyde from a standard graph.

α,β-UNSATURATED ALDEHYDES AND KETONES

Members of the α-β-unsaturated aldehydes and ketones form hydrazones with Girard-T reagent, trimethylacethydrazide ammonium chloride.

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[50] G. R. Primavesi, *Analyst* **78**, 647-51 (1953).

This is particularly applicable to citral and cinnamaldehyde in neutral solution and to carvone in the presence of acetic acid. The hydrazones are water-soluble and have a maximum at a longer wave length in the ultraviolet than the semicarbazones.[51] All three obey Beer's law. For more details see the specific compound.

Procedure—*By Girard-T reagent. Citral and cinnamaldehyde in essential oils.* Select a sample of oil according to Table 17. Reflux for 12 minutes with 5 ml. of 95% ethanol and 0.2 gram of Girard-T reagent. Cool and transfer to a separatory funnel with 50 ml. of ether. Rinse the reaction flask with 25 ml. of water and add to the separatory funnel. Shake gently, separate, and withdraw the aqueous layer. Rinse the reaction flask and ether layer with two 10-ml. portions of water.

TABLE 17. CONDITIONS FOR DETERMINATION OF CITRAL, CINNAMALDEHYDE, AND CARVONE BY GIRARD-TREAGENT

Oil	*Quantity* (mg.)	*Dilution* (ml.)	λ *max.* (mμ)	*E*(1 per cent, 1 cm.)
Citral	10-15	10 to 100	281	1937
Lemon	300	10 to 100	281	
Lemon grass	15-20	10 to 100	281	
Cinnamaldehyde	10-15	10 to 250	313	3180
Cinnamon bark	20-30	10 to 250	313	
Cinnamon leaf	200	10 to 250	313	
Carvone	15-20	10 to 100	272.5	1267
Dill	20	10 to 100	272.5	
Caraway	20	10 to 100	272.5	

Dilute the combined aqueous layers to 500 ml. Dilute an aliquot according to Table 17 and read at 281 mμ for citral and 313 mμ for cinnamaldehyde. If both are present simultaneous equations must be used.

Carvone. Reflux an amount of oil according to Table 17 for 70 minutes with 5 ml. of 95% ethanol, 0.5 ml. of glacial acetic acid, and 0.2 gram of Girard-T reagent. Transfer to a separatory funnel containing sufficient 4% sodium hydroxide to neutralize 0.45 ml. of glacial acetic acid using 50 ml. of ether. Complete as for citral and cinnamaldehyde from "Rinse the reaction flask with. . . ." Read at 272.5 mμ.

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[51] J. B. Steulake and W. D. Williams, *J. Pharm. and Pharmacol.* 9, 900-7 (1957).

CITRAL

Citral, the aldehyde in lemon oil responsible for the characteristic lemon odor, forms a green complex with vanillin and piperidine with an absorption maximum at 605 mμ.[52] Cf. Vol. III, p. 267. Saturated aldehydes produce a yellow color with the reagent. α,β-Unsaturated aldehydes produce a red color while citral, dihydrocitral, and pseudoionone give a green color. The latter compounds have a branching methyl group on the terminal carbon atom of a double bond system conjugated with the carbonyl group. Therefore only dihydrocitral and pseudoionone interfere. Compounds commonly present in synthetic vanillin, such as isovanillin, vanillic acid, and guiacol, do not interfere. A large excess of vanillin over citral is required for color development. Dilution of the reagent with water or acid changes the green color to yellow, while dilution of the color complex with ethanol causes rapid color fading, which levels off after 20 minutes.

Citral has an absorption maximum at 238 mμ[53] (Cf. Vol. III, pp. 267-8). For determination with Girard-T reagent see page 261.

Procedure—*Oils.* Dilute a lemon oil sample 500:1 and develop a 2-ml. aliquot. Dilute an orange oil sample 25:1 and develop a 2-ml. aliquot. If smaller aliquots are taken, dilute to 2 ml. with ethanol.

To an aliquot containing 0.0152-0.152 mg. of citral, add 4 ml. of a mixture of equal volumes of 15.215% vanillin solution in absolute ethanol and 7% piperidine solution in absolute ethanol or methanol. Stopper, shake, and read after 55 minutes at 605 mμ. Complete the determination within 100 minutes after addition.

FURFURAL

The red color which develops when furfural is mixed with aniline acetate (Vol. III, p. 274) is based on the reaction of furfural with aniline to form a colored Schiff's base. This is applicable to furfural in lubricating oil.[54] Since the color is unstable, the reaction mixture must be transferred immediately to a photometer cell and the color development followed quantitatively by continually adjusting the instrument to read the maximum absorbance. Parallel-faced rather than cylindrical cells should be used to minimize differences in the refractive indices of the benzene and glacial acetic acid, and the light source of the instrument should be adjusted so that equal readings are obtained with benzene and glacial acetic acid. Under these conditions, colored samples can be used if a correction is applied. The color is modified by substituents on the benzene neucleus. The curves have the same shape with the maximum for *o*-toluidine, *p*-toluidine, *p*-phenetidine, and 1-naphthylamine falling at 430-570 mμ.

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[52] W. L. Stanley, R. C. Lindwall, and S. H. Vannier, *J. Agr. Food Chem.* 6, 858-60 (1958).

[53] K. L. Surve, K. K. Chakravarti, and S. C. Bhattacharyka, *Perfumery Essent. Oil Record* 49, 624-6 (1958).

[54] O. I. Milner and D. Liederman, *Anal. Chem.* 27, 1822-3 (1955); A. N. Ponomarev, *Zavodskaya Lab.* 22, 289-92 (1956); O. T. Khachidze, *Sadovodstvo. Vinogradarstvo i Vinodelie Moldavii* 12, No. 1, 32-3 (1957); D. A. Isăcescu, S. Biller, and

Although methylfurfural and hydroxyfurfural react with the reagent, they absorb only very slightly at the wave length used in this technic. Formaldehyde, acetaldehyde, propionaldehyde, and crotonaldehyde have no effect on the results. The absorption coefficient for the aniline acetate reagent should be established before the test by dividing the instrument reading of a standard solution into the furfural content.

An alternative is to extract the furfural from raffinate by aqueous sodium bisulfite.[55] Oxidation of the bisulfite by iodine then liberates the furfural. After removal of excess iodine by dropwise addition of thiosulfate the furfural is determined by aniline acetate. *p*-Anisidine and *p*-bromoaniline are similarly used.[56]

2,4-Dinitrophenylhydrazine in 1:5 hydrochloric acid is also a reagent for furfural.[57] The technic is a simple one of passing air through or over the sample and then over a paper strip soaked in a saturated solution of 2,4-dinitrophenylhydrazine in 1:16 hydrochloric acid. Determination is by comparison by eye with standard strips.

Anthranilic acid is applied to develop a color in dilute ethanolic solution.[58] Dinitrophenylhydrazine is a reagent for nephelometric estimation in aqueous solution at 0.0015-0.015 mg. per ml.[59]

Furfural is read directly at 277.5 mμ.[60] Serious interferences are by bis(5-formyl-2-furfuryl)ether, and 5-hydroxymethyl-2-furfural. Less se-

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M. Macavei-Bestelei, *Acad. rep. populare Romîne, Studii cercetări chim.* **6**, 247-61 (1957). Cf. D. A. Isăcescu and S. Biller, *Ibid.* **1**, 193-203 (1958).

[55] W. C. Woelfel, W. D. Good, and C. A. Neilson, *Petroleum Engr.* **24**, No. 7, C42-44 (1952).

[56] F. Aeschlimann, P. O. Bettige, and J. H. Eggers, *Z. anal. Chem.* **161**, 324-44 (1958).

[57] A. R. Javes, *Proc. Am. Petroleum Inst. III* **29 M,** 39-41 (1949).

[58] D. A. Isăcescu, S. Biller, and M. Macavai-Bestelai, *Commum. acad. rep. populare Romîne,* **8**, 897-901 (1958).

[59] M. Rothe and I. Voigt, *Ernährungsforschung* **2**, 444-53 (1957).

[60] G. Mackinney and O. Temmer, *J. Am. Chem. Soc.* **70**, 3586-90 (1948).

rious interferences are by 5-hydroxymethyl-2-furoic acid and 2,5-bis-hydroxymethylfuran.[61]

The steam-distillation of darkened dried apricot extract give furfural and hydroxymethylfurfural. They are also separable from the sample by ether extraction. In either case they are read at 280 and 285 mμ to give approximate determinations.[62]

Sodium bisulfite and furfural react in lubricating oil to form an addition compound from which furfural can be regenerated by addition of alkali and read at 277 mμ.[63] This permits the determination of concentrations of furfural in the range of 0.0002-1%. Standards must be prepared from freshly distilled furfural or from furfural in isooctane. Excess potassium hydroxide is added since sodium bisulfite interferes. Oil samples are dissolved in isooctane to reduce their viscosity to permit extraction of the furfural.

For development of furfural by 2-thiobarbituric acid see page 177. For determination of furfural by thymol see page 197.

Sample—*Oil.* A satisfactory sample in the range of 0.0002-1% of furfuraldehyde is 0.1-25 grams. Dilute with 50 ml. of isooctane to reduce the viscosity and permit extraction. Develop by formation of the sodium bisulfite-furfural addition complex.

Procedure—*By aniline acetate. Colored samples.* Dissolve a weighed sample in benzene and dilute to 100 ml. with benzene. Take three readings as follows. (A) Dilute an aliquot containing 0.01-0.06 mg. of furfural to 50 ml. with benzene and read at 520 mμ against benzene. (B) Dilute a similar aliquot to 20 ml. with benzene and add 25 ml. of 10% aniline acetate solution in glacial acetic acid. Dilute to 25 ml. with benzene. Transfer within 1 minute using benzene as a blank. Read at 520 mμ at 10-15 second intervals. Record the reading when the maximum absorbance is reached in approximately 1-2 minutes. (C) Dilute 25 ml. of 10% aniline acetate solution in glacial acetic acid to 50 ml. with benzene and read at 520 mμ against benzene. Calculate the corrected instrument reading as follows:

$$R_C = B - (C + A).$$

Uncolored samples. Follow the procedure for colored samples but omit readings A and C.

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[61] J. H. Turner, P. A. Rebers, P. L. Barrick, and R. H. Cotton, *Anal. Chem.* 26, 898-901 (1954).

[62] Abdul Wahhab, *Pakistan J. Sci. Research* 4, 111-13 121 (1952).

[63] L. L. Gent, R. C. Pomatti, and Henry Levin, *Anal. Chem.* 26, 413-4 (1954).

By the sodium bisulfite addition product. Oil. To an aliquot of oil sample containing 0.0002-1% of furfural diluted to 50 ml. with isooctane, add 20 ml. of 10% sodium bisulfite solution. Shake vigorously for 5 minutes and let stand for the layers to separate. To 10 ml. of the aqueous layer containing the sodium bisulfite-furfural addition product add 15 ml. of 10% potassium hydroxide solution and dilute to 50 ml. with water. Read at 277 mμ against water. Subtract the absorbance of a reagent blank.

BUTADIENE-FURFURAL

By the formation of a 2,4-dinitrophenylhydrazone and its extraction into isooctane, 2,3,4,5-bis(Δ^2-butenylene) tetrahydrofurfural, a butadiene-furfural condensation product, used as a fly repellent, is determined.[64] The excess reagent, which absorbs in the same region as the dinitrophenylhydrazone product remains in the aqueous phase during extraction, thus eliminating the need for adding alkali to shift the absorbance of the dinitrophenylhydrazone into the visible region. Use of alkali for this purpose does not always give reproducible results and the color is more likely to fade rapidly.

The sensitivity can be increased by concentrating the carbonyl compound in the isooctane solvent before analysis. Since the calibration curve is independent of reagent concentration if a simultaneous blank determination is made, one calibration curve is sufficient for a number of samples. This extraction procedure may be adaptable for other carbonyl compounds which form similar hydrazones.

Procedure—To prepare the reagent, mix one volume of a saturated solution of 2,4-dinitrophenylhydrazine in carbonyl-free ethanol with 3 volumes of 1:2 phosphoric acid. Add a 10-ml. sample containing 0.01-0.2 mg. of the test substance in isooctane to 10 ml. of the reagent solution. Mix continuously for 30 minutes. Remove the reagent phase and wash the octane phase for 10 minutes with an equal volume of 1:2 phosphoric acid. Read the octane phase at 340 mμ against isooctane similarly treated.

5-HYDROXYMETHYL-2-FURFURALDEHYDE

A color of this aldehyde is developed with *p*-toluidine and barbituric acid or ferrocyanide and zinc acetate.[65] If read directly 5-hydroxy-2-fur-

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[64] P. E. Toren and B. J. Heinrich, *Ibid.* 27, 1986-8 (1955).

[65] O. Winkler, *Z. Lebensm.-Untersuch. u. -Forsch.* 102, 161-7 (1955).

furaldehyde has a maximum at 284 mμ.[66] The maximum of bis(5-formyl-2-furfuryl) ether almost coincides. Furfural, interferes seriously; 5-hydroxymethyl-2-furoic acid and 2,5-bishydroxymethylfurane slightly.[67]

To apply corrections for interfering substances, a hydrolyzed sirup containing 5% of starch is read at 245 and 284 mμ. The difference in the readings is a measure of hydroxymethylfurfural.[68] Wood hydrolysates after steam distillation of the furfural are similarly read at 243 and 275 mμ. For development by phenol in sulfuric acid see page 167.

Procedure—*Honey.* To 2 ml. of 17% honey sample in water add 5 ml. of a 10% solution of *p*-toluidine in 10% acetic acid in ispropanol. Add 1 ml. of 0.5% barbituric acid in water and mix. Read after 3-4 minutes at 550 mμ against a sample blank.

Alternatively, dilute 10 ml. of a 17% aqueous solution of honey with 50 ml. of water. Add 1 ml. of 15% solution of potassium ferrocyanide and 1 ml. of 30% zinc acetate dihydrate solution. Filter and discard the first 20 ml. of filtrate. Read at 245, 285, and 325 mμ. Subtract the readings at 295 and 325 mμ from that at 285 mμ as a measure of hydroxymethylfurfural.

GLYCOLALDEHYDE

The reaction product between glycoaldehyde and 2,4-dinitrophenylhydrazine is an osazone, glyoxal bis(2,4-dinitrophenylhydrazone) which shows maximum absorption at 600 mμ.[69] Other carbonyl compounds do not interfere since they do not absorb at 600 mμ. Beer's law is followed for 0.005-0.045 mg. A green color is formed when glycoaldehyde is heated with naphthoresorcinol in 2:1 sulfuric acid.[70] The intensity at 660 mμ is proportional to the concentration. The interference of hydroxypyruvate is removed by adsorption on BioDeminrolit G. Dihydroxyfumaric acid interferes. The corresponding derivative of glyoxal is indistinguishable.

Procedure—*By 2,4-dinitrophenylhydrazine.* To an aqueous sample containing 0.005-0.045 mg. of glycoladehyde add an excess of 2,4-dinitrophenylhydrazine solution in 1:10 hydrochloric acid. Dissolve any crystalline precipitate by adding 5 ml. of 4% sodium hydroxide solution and sufficient 95% ethanol to dilute to 100 ml. Read at 600 mμ.

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[66] R. B. Wearn, W. M. Murray, Jr., M. P. Ramsey, and N. Chandler, *Anal. Chem.* **20**, 922-4 (1948).

[67] J. H. Turner, P. A. Rebers, P. L. Barrick, and R. H. Cotton, *Ibid.* **26**, 898-901 (1954).

[68] Erich Lindemann, *Die Starke* **7**, 280-4 (1955).

[69] C. Neuberg and E. Strauss, *Arch. Biochem.* **7**, 211-30 (1945); *Ibid.* **11**, 457-65 (1946); T. Banks, C. Vaughn, and L. M. Marshall, *Anal. Chem.* **27**, 1348-9 (1955).

[70] D. F. Dickens and D. H. Williamson, *Biochem. J.* **68**, 84-8 (1958).

GLYOXAL, ETHANEDIAL

Glyoxal reacts with dianilinoethane to form 1,1′-3,3′-tetraphenyl-2,2′-biimidazolidine. This produces a blue color when heated with hydrochloric acid.[71] The method is specific for glyoxal. Sensitivity is increased by increasing the ratio of dianilinoethane to glyoxal and by conducting the reaction in boiling ethanol rather than in water. The color formation depends on acidity and time of reaction. The peak absorbance is reached in 30 minutes although the color is stable for 2 days. Large amounts of anhydrolyzed oxystarch interfere through the production of glyoxal during the color development. Reducing sugars, formaldehyde, and acetaldehyde do not interfere.

The reaction of glycolic aldehyde with 2,4-dinitrophenylhydrazine is applicable to glyoxal as is also that in sulfuric or acetic acid with 2,3-diaminothiazine.[72]

Procedure—To a 3-ml. aliquot of the sample containing approximately 1.2 mg. of glyoxal, add 3 ml. of 1.33% 1,2-dianilinoethane dihydrochloride solution in 95% ethanol. Add 0.6 ml. of concentrated hydrochloric acid, cover and heat at 77° for 30 minutes. Dilute to 10 ml. with 95% ethanol. Read at 550 mμ against a reagent blank. Use a glyoxal-sodium bisulfite monohydrate solution as a reference standard. The color is constant for at least 2 days.

OXYMETHYLENEGLYCOLALDEHYDE

Folin's reagent, a mixture of phosphomolybdic and phosphotungstic acids is used for the photometric determination of oxymethyleneglycolaldehyde.[73] Beer's law is followed.

Procedure—Use a sample deproteinized with phosphotungstic acid containing 0.005-0.1 mg. per ml. of oxymethyleneglycolaldehyde. Add 1 ml. of phosphate buffer for pH 6.2 (Vol. I, p. 174) 0.1 ml. of 18.6% iodoacetamide solution, 0.1 ml. of 0.025% copper sulfate pentahydrate solution, and 3 ml. of water. After 3 minutes heat with 0.5 ml. of Folin's reagent (Vol. III, p. 116) at 100° for 3 minutes. Read the blue color after 15 minutes at 730 mμ or 875 mμ.

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[71] H. W. Wanzlick and W. Lochel, *Chem. Ber.* **86**, 1463-6 (1953); C. S. Wise, C. L. Mehltretter, and J. W. Van Cleve, *Anal. Chem.* **31**, 1241-2 (1959).

[72] J. M. De Charz, E. Kun, and H. C. Pitot, *Anal. Chem.* **26**, 449-52 (1954).

[73] Susumu Adachi, *Nippon Nôgei-kagaku Kaishi* **31**, 93-7 (1957).

CINNAMIC ALDEHYDE, CINNAMALDEHYDE

Salicylaldehyde in the presence of alcoholic potassium hydroxide solution is a reagent for cinnamic aldehyde.[74] (Cf. Vol. III, pp. 278-80.) The cinnamic aldehyde is extracted from the sample with diethyl ether to eliminate interference of eugenol. For determination by Girard-T reagent see page 261. For development by *p*-aminophenol in 6% trichloroacetic acid see page 259.

Samples—*Cinnamon oil.* Dissolve a 0.1-gram sample in 5 ml. of diethyl ether. Shake with 10 ml. of 10% potassium hydroxide solution. Wash the aqueous-alkaline layer with two successive 5-ml. portions of ether. Discard the aqueous layer. Wash the ether layers successively with 10% potassium hydroxide solution and discard these washings. Eugenol has been removed. Combine the ether extracts and distil off the ether. Dissolve the residue in 5 ml. of 95% ethanol for development.

Cinnamon bark. Distil a 10-gram coarsely ground sample with 200 ml. of water and collect 160 ml. of distillate. Shake with 50 ml. of diethyl ether. Separate and extract the aqueous layer with 50 ml. of ether. Extract the combined ether extracts with three 100-ml. portions of 10% potassium hydroxide solution. Wash with alkaline extract successively with 50 ml. of ether. Combine the ether layers and evaporate the ether. Dissolve the residue in 5 ml. of 95% ethanol for development.

Procedure—Reflux the prepared sample in 95% ethanol with 10 ml. of 10% salicylaldehyde in 95% ethanol for 1 hour. After cooling add 10 ml. of amyl alcohol and 70-80 ml. of saturated salt solution. Shake thoroughly and withdraw the aqueous layer. Extract the aqueous layer with 10 ml. more of amyl alcohol. Discard the aqueous layer and combine the amyl alcohol extracts. Wash this with 30-40 ml. of saturated salt solution. Filter and wash the filter with amyl alcohol, adjusting the filtrate to a known volume. Read at 624 mμ.

VANILLIN

In the determination of vanillin by direct reading (Vol. III, p. 280) there is a change in absorption with changes in pH of the solution in the same way that acid-base indicators change with varying pH.[75] Adjustment of the pH to values above 9.6 or below 5 is appropriate for the quantitative analysis of vanillin based on absorbancies of the basic and acid forms respectively. The absorption maximum for the basic form of vanillin is shifted from 353 mμ for the alcoholic solution to 347 mμ for the aqueous solution.

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[74] S. Collett, *Mfg. Chemist* 23, 96-8, 411-2, 421 (1952).

Phosphotungstic-phosphomolybdic acid is the reagent for an official method of determination of vanillin[76] (Vol. III, p. 283). Beer's law may not necessarily be followed. Thiobarbituric acid reacts with vanillin to yield a yellow complex with an absorption peak at 432-4 mμ which obeys Beer's law.[77] The color intensity is directly related to the concentration of phosphoric acid in the reaction mixture.

For separation of vanillin from ethyl vanillin and coumarin by chromatography see page 121. For its determination by 2,4-dinitrophenylhydrazine see page 255. Dinitrophenylhydrazine is a reagent for nephelometric estimation in aqueous solution at 0.0015-0.015 mg. per ml.[78]

Sample—*Vanilla extract.* Dilute a 2-ml. sample containing up to 20 mg. of vanillin to 80 ml. with water. If the vanillin concentration exceeds 1 gram per 100 ml. of sample, further dilute a 50-ml. sample to 100 ml. with water and use this diluted sample.

To the sample, add 2 ml. of a filtered lead acetate solution containing 50 grams each of a neutral and basic lead acetate in 1 liter of water. Dilute to 500 ml. with water, mix, and filter, discarding the cloudy portion of the filtrate. Develop by phosphotungstic-phosphomolybdic acid reagent or adjust to read in the ultraviolet.

Procedure—*In the ultraviolet.* Mix 10 ml. of sample solution with 2 ml. of 0.4% sodium hydroxide solution and dilute to 100 ml. with water. The pH must be above 10.5. Read at 347 mμ.

By phosphomolybdic-phosphotungstic acid. To prepare the reagent reflux for 2 hours, 750 ml. of water, 100 grams of sodium tungstate, 20 grams of phosphomolybdic acid, and 50 ml. of phosphoric acid. To a 10-

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[75] D. T. Englis and L. A. Wollermann, *Anal. Chem.* 29, 1151-3 (1957).

[76] "Official Methods of Analysis of the Association of Official Agricultural Chemists," 8th ed., pp. 319-20, Association of Official Agricultural Chemists, Washington, D. C. (1955).

[77] N. S. Kapur, W. M. Narayana, G. S. Bains, and D. S. Bhatia, *Chem. & Ind. (London)* 1957, 1272-3; G. S. Bains, N. S. Kapur, and D. S. Bhatia, *J. Sci. and Ind. Research (India)* 17B, 462-7 (1958).

[78] M. Rothe and I. Voigt, *Ernährungsforschung* 2, 444-53 (1957).

ml. clear aliquot of sample containing up to 20 mg. of vanillin, add 5 ml. of reagent. Mix and let stand for 5 minutes. Add 10 ml. of 25% sodium carbonate solution, mix, and let stand for 10 minutes. Dilute to 100 ml. with water, mix, and filter, discarding the cloudy portion of the filtrate. Read immediately at 610 mμ.

By thiobarbituric acid. To a sample containing 0.005-0.22 mg. of vanillin per ml., add 2 ml. of 0.36% thiobarbituric acid solution in 9.8% phosphoric acid. Dilute to 10 ml. and read at 432 to 434 mμ.

NITROFURAZONE, 5-NITRO-2-FURALDEHYDE SEMICARBAZONE

The increasing use of nitrofurazone in the prophylaxis of coccidiosis in poultry makes necessary its determination in commercial feeds. An important reaction is the formation of a bright red 5-nitro-2-furaldehyde phenylhydrazone, which can be extracted and measured.[79] The absorbance is measured before and after reduction of an aliquot with sodium hydrosulfite as a measure of nitrofurazone. If an untreated sample is available, this may be used in place of the reduced aliquot as a blank, thus eliminating the reduction step.

Other materials commonly found in poultry feeds which do not interfere include enheptin, nicarbazin, chlortetracycline, chloramphenicol, oxytetracycline, nitrophenide, arsanilic acid, 3-nitro-4-hydroxyphenylarsonic acid, *p*-aminobenzoic acid, sulfaquinoxaline, thiouracil, phenothiazine, penicillin G, dihydrostreptomycin sulfate, streptomycin-calcium chloride, and bacitracin. Riboflavin is converted to flavin under the conditions of the method and does not interfere. If a high background absorbance is evident, a pre-extraction may be necessary.

Another method for nitrofurazone is based on the development of a pink color on treatment with strong sodium hydroxide solution.[80] Interferences are removed by pre-extraction with heptane and carbon tetrachloride. Then it is extracted with dioxane. The pink color fades 5% after 1 minute at temperatures of up to 20°, 10% after 2 minutes, and 15% after 5 minutes.

Samples—*Feeds and premixes containing up to 0.022% of nitrofurazone.* A suitable sample containing 0.5-1 mg. of nitrofurazone is approximately 9.1 grams for 0.0055% and 0.011% feeds and 4.55 grams for 0.022% feeds. Do not use a sample less than 4.55 grams. Grind to 30-mesh or finer and add 70 ml. of absolute ethanol. Stopper and shake in subdued light at 55-60° for 1 hour. Filter, wash with absolute ethanol, and dilute the filtrate and washings to 100 ml. with absolute ethanol. Dilute 25 ml. of the extract with an equal volume of water. Reduce a 25-ml. aliquot by adding 25 mg. of sodium hydrosulfite and shaking occasionally for 15 minutes, to obtain a correction blank by destroying the nitrofurazone. If a control feed sample is available, omit the reduction step and prepare the blank from the control sample. Develop by phenylhydrazine.

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[79] V. R. Ellis, E. S. McKay, and H. E. Paul, *J. Assoc. Offic. Agr. Chemists* 36, 417-21 (1953); J. A. Buzard, V. R. Ellis, and M. F. Paul, *Ibid.* 39, 512-8 (1956); J. A. Buzard, D. M. Vrablic, and M. F. Paul, *Antibiotics and Chemotherapy* 6, 702-7 (1956).

[80] Sybil S. Thompson, *Analyst* 81, 443-4 (1956).

Feeds and premixes containing 0.022-0.1% of nitrofurazone. Treat a 4.55-gram sample with enough absolute ethanol so that the nitrofurazone concentration in the extract is approximately 0.5-1 mg. per 100 ml. The range will be about 75 to 300 ml. Follow the procedure for concentrations up to 0.022% nitrofurazone starting with ". . . Stopper and shake in subdued light."

Feeds and premixes containing 0.1-11% of nitrofurazone. For each gram of sample add 11 ml. of *N,N'*-dimethylformamide and shake for 10-15 minutes. Decant the extract and centrifuge. Dilute with 50% ethanol to give approximately 0.004 mg. of nitrofurazone per ml. Continue as in the determination of concentrations up to 0.022% nitrofurazone starting with ". . . Dilute 25 ml. of the extract with an equal volume of water."

Poultry feeds containing up to 0.005% of nitrofurazone. Dry a 5-gram finely ground sample in a vacuum oven at 100° for 30 minutes. Extract in a Soxhlet extraction thimble for 3 hours with 40 ml. of heptane and for 1 hour with 40 ml. of carbon tetrachloride. Let dry and add 25 ml. of dioxane. Filter after 1 hour away from strong light with occasional agitation. Measure the volume of the filtrate, and add an equal volume of water. The filtrate will become cloudy. Mix and filter. Develop with sodium hydroxide.

Procedure—*By phenylhydrazine.* Add 8 ml. of prepared sample to a fresh mixture of 1 ml. of 1.5% phenylhydrazine hydrochloride solution and 1 ml. of 3:1 hydrochloric acid. Mix and heat for 25 minutes at 70°. Cool at 15° for 5 minutes and add 10 ml. of toluene. Stopper and shake vigorously for 20-30 minutes. Centrifuge a portion of the toluene layer and read at 440 mμ against toluene. Correct for the reduced or untreated control correction blank.

By sodium hydroxide. To a 5-ml. aliquot, containing up to 0.005% of nitrofurazone, add in the photometer cell 1 ml. of 10% sodium hydroxide solution in 50% ethanol. Stir and read immediately after air bubbles disappear.

FURAZOLIDONE, *N*-(5-NITRO-2-FURFURYLIDENE)-3-AMINO-2-OXAZOLIDONE

Furazolidone is used in poultry feeds for control of fowl typhoid, pullorum, paratyphoid, blackhead, histomoniasis, hexamitiasis, and salmonelloses. When dissolved in anhydrous isopropanol furazolidone is read directly at 357 mμ. Ethanol has also been used as an extracting solvent. It has the advantage of extracting a greater quantity of drug but it extracts more interfering substances than isopropanol does.[81] Pre-extraction with hexane removes many interfering substances. The solutions should be protected from strong light, sunlight, and fluorescent light, and should not be dried in ovens. Readings are corrected by use of a reduced sample or with an untreated sample as a blank. Furazolidone is also determined as the phenylhydrazone, page 271.

Samples—*Feeds.* Prepare a 6 × 3/4 inch chromatographic column with a 5-mm. hole at the bottom containing the following: a compressed 8-mm. cotton plug at the bottom, an 8-mm. layer of diatomaceous earth filter aid, a sample containing 1 mg. of furazolidone. Tramp down firmly and add another cotton plug at the top. Heat hexane to near boiling and fill the column. Allow the solvent to pass through by gravity. If there is any solvent left after 10 minutes, apply gentle suction. Use a total of four 15-ml. portions of solvent. Draw air through the tube to dry completely and let it return to room temperature.

Empty the column by pushing a rod through the bottom and add exactly 100 ml. of anhydrous isopropanol to the entire contents. Cover stoppers with aluminum and agitate for 55 minutes in a bath at 60° to insure complete solution of the furazolidone. Filter and read in the ultraviolet.

Feeds containing up to 0.022% of furazolidone. Follow the procedure under nitrofurazone, substituting a 3-hour extraction time for the one hour in the procedure. Read as the phenylhydrazone.

Feeds containing up to 0.022-0.1% of furazolidone. Follow the procedure for the corresponding concentration of nitrofurazone, making the substitution of a 3-hour extraction time for one hour. Read as the phenylhydrazone.

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[81] Chester A. Luhman, *J. Assoc. Offic. Agr. Chemists* 39, 313-8 (1956).

Procedure—*In the ultraviolet.* If the sample is available both treated with furazolidone and untreated read the sample. If treated sample must be used as blanks, dilute two 20-ml. aliquots of the prepared isopropanol solution with 10 ml. of water. Add 1 mg. of sodium hydrosulfite per ml. of solution to one aliquot as a blank and let it stand with occasional agitation for at least 20 minutes. Read the sample and blank at 370 mμ and subtract the absorbance of the reduced blank from the absorbance of the unreduced sample.

SALICYLIC ALDEHYDE

Salicylic aldehyde is determined by its fluorescence when treated with aluminum chloroisopropylate in ethanol.[82] Salicylic acid and methyl salicylate also fluoresce and therefore interfere. Acetylsalicylic acid gives no fluorescence with the cold reagent but reacts when heated. Salicylic acid amide shows fluorescence but it is not proportional to the concentration.

Procedure—To a solution containing 0.005-0.015 mg. of salicylic aldehyde in absolute ethanol, add 1 ml. of 3.5% aluminum chlorisoproplate solution in 95% ethanol. Dilute to 10 ml. with 95% ethanol. Let stand for 5 minutes and read the fluorescence against a reagent blank.

p-AMINOSALICYLIC ALDEHYDE

p-Aminosalicylic aldehyde is determined by thymol.[83]

Procedure—*Cerebrospinal fluid.* Deproteinize a 3-ml. sample with 1.5 ml. of 10% trichloroacetic acid solution. Shake and filter. Cool a 3-ml. deproteinized sample to 0° and add 0.5 ml. of 1:5 hydrochloric acid. Shake and add 0.25 ml. of 0.1% sodium nitrite solution. Let stand for 3 minutes, remove from the ice bath, and add 0.25 ml. of 10% urea solution. Add 1 ml. of 1% thymol solution in 30% sodium hydroxide solution. Shake for several minutes and read immediately at 465 mμ.

α-THUJONE

α-Thujone is determined by 3,5-dinitrobenzoic acid in the presence of sodium hydroxide. β-Thujone interferes since it reacts to a maximum color within 3 minutes under the specified conditions.[84]

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[82] I. Simonyi and G. Tokár, *Magyar Kém. Folyóirat* **63,** 210-3 (1957).

[83] G. Orestano, M. Ghione, and B. Chiarlo, *Arch. ital. sci. farmacol.* (3), **4,** 369-73 (1954).

[84] D. H. E. Tattje, *Pharm. Weekblad* **93,** 689-94 (1958).

Procedure—To 4 ml. of solution in 95% ethanol containing up to 3.3 mg. of α-thujone, add 5 ml. of 4% 3,5-dinitrobenzoic acid solution in 95% ethanol and 3 ml. of 16% sodium hydroxide solution. Read at 537.5 mμ after 11 minutes.

DIALDEHYDE

A determination of periodate-oxidized starch, oxystarch, is based on a measurement of carbonyl groups by converting them to insoluble nitrophenylhydrazones which produce a yellow color in ethanol.[85] The color is constant for 3 days as compared to the violet color produced in ethanolic sodium hydroxide[86] which is not stable. The method is applicable to oxystarches of low dialdehyde content. Substances which form insoluble *p*-nitrophenylhydrazones with the reagent interfere. The absorbance deviates slightly from Beer's law, especially above a value of 0.7. The oxystarch is calculated on the basis that 100% oxystarch would have an absorbance of 467.7 per mg. per ml.

Procedure—*Oxystarches containing less than one dialdehyde unit per hundred repeating units.* To a sample equivalent to 0.25 mg. of 100% oxystarch, add 20 ml. of water and heat at 100° for 30 minutes, agitating occasionally. Add 1.5 ml. of a reagent prepared by dissolving 250 mg. of *p*-nitrophenylhydrazine in 15 ml. of glacial acetic acid. Heat for 1 hour with occasional agitation to complete the formation of the red *p*-nitrophenylhydrazone. Cool, add 0.4 gram of a filter aid, and vacuum-filter on a fritted glass funnel. Wash with two 5-ml. portions of 7% acetic acid followed by two 5-ml. portions of water. Rinse the test tube, pour the rinse into the funnel, and discard the washings. Repeatedly wash the test tube and funnel with hot 95% ethanol solution until all the precipitate is dissolved and the solution is collected in a receiving flask. Dilute to 250 ml. with 95% ethanol, and read at 445 mμ against a blank similarly prepared using starch in place of oxystarch.

Oxystarches containing more than 1 dialdehyde unit per hundred repeating units. To dissolve a highly oxidized sample, add 50-62 mg. of oxystarch to 180 ml. of water and heat with occasional agitation for 2-3 hours. Take an aliquot equivalent to 5 mg. of 100% oxystarch. If the sample is not difficult to dissolve, add immediately to 20 ml. of water. Follow the procedure for samples containing less than one dialdehyde unit per hundred repeating units, using 3 ml. of the reagent solution described in the procedure. Dilute a 5-ml. aliquot from the final 250 ml. to 100 ml. with 95% ethanol before reading.

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[85] C. S. Wise and C. L. Mehltretter, *Anal. Chem.* 30, 174-5 (1958).

[86] C. Neuberg and E. Strauss, *Arch. Biochem.* 7, 211-30 (1945).

CHAPTER 12

KETONES

KETONES having multiple hydroxyl groups do not appear here but under the appropriate sugar classifications in Chapters 7, 8, and 9.

In approximate determination of mixed ketones a reading on the mixed 2,4-dinitrophenylhydrazones is applicable. Thus 8 ketones from acetone to methylnonyl ketone have an average extinction coefficient of 1.87 at 425 mμ and 0.888 at 530 mμ, Table 16. The corresponding average values for 8 aldehydes are 2.13 at 425 mμ and 0.871 at 530 mμ.

The extinction coefficient of the 2,4-dinitrophenylhydrazone of methyl ethyl ketone is 1.79 at 425 mμ. The extinction coefficient of diethyl ketone as the 2,4-dinitrophenylhydrazone is 1.81 at 425 mμ and 0.863 at 530 mμ. The extinction coefficient of methyl isopropyl ketone as the 2,4-dinitrophenylhydrazone is 1.88 at 425 mμ and 0.863 at 530 mμ. The extinction coefficient of di-*n*-propyl ketone as the 2,4-dinitrophenylhydrazone is 1.89 at 425 mμ and 0.923 at 530 mμ. The extinction coefficient of methyl *n*-butyl ketone as the 2,4-dinitrophenylhydrazone is 1.86 at 425 mμ and 0.870 at 530 mμ. The extinction coefficient of di-*n*-butyl ketone as the 2,4-dinitrophenylhydrazone is 1.85 at 425 mμ and 0.913 at 530 mμ. The extinction coefficient of methyl *n*-hexyl ketone as the 2,4-dinitrophenylhydrazone is 1.91 at 425 mμ and 0.910 at 530 mμ. The extinction coefficient of methyl *n*-nonyl ketone as the 2,4-dinitrophenylhydrazone is 1.90 at 425 mμ and 0.873 at 530 mμ.

For determination of β-(*p*-toluidino)propenyl ketone after hydrolysis to acetylacetone see page 282. β-Ionone forms a red color when heated for 1 hour at 40° with acetic acid and hydrochloric acid. Beer's law is followed for 1-5 mg. at 550 mμ. α-Ionone does not develop a color under these conditions.[1] Phenylacetone in amphetamine is determined by direct reading of amphetamine in ethanol at 290 mμ.[2] For determination of butanone by oxidation to iodoform and reading in the ultraviolet see page 276.

For determination of 2-pentanone, 2-hexanone, 2-heptanone, or 2-octanone by oxidation to iodoform and reading in the ultraviolet see page 276. Ferric ion and tropolone, 2-hydroxy-3,4,6-cycloheptatrien-1-one, give a green 1:1 complex in the presence of excess ferric ion.[3] The maximum absorption is at 435 mμ and the maximum color developed at pH 0.5-1. A solution of *p*-hydroxypropiophenone in ethanol is appropriate for reading at 5 ppm. Maxima are at 220 and 275 mμ.[4] Phenobarbital does not interfere but methylthiouracil does to some extent. Pulegone is read at 255 mμ.[5]

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[1] Maurice Pesez, *Bull. soc. Chem. France* **1955, 190.**

[2] Stanley K. Freeman, *J. Am. Pharm. Assoc.* **42,** 621-2 (1953).

[3] Y. Oka and S. Matsuo, *Sci. Repts. Research Insts., Tohoku Univ.* **A10,** 299-305 (1958).

Cyclohexanone is determined by reaction with the diazonium salt of *H*-acid to give a cherry-red diazo dye with its maximum absorption at 550 mμ.[6] Sulfite is present to prevent self-coupling of the diazotized *H*-acid. The presence of acetic anhydride and urotropine prevents oxidation of cyclohexanol to cyclohexanone. For its determination by *p*-hydroxybenzaldehyde see page 44. Methylethyl ketone and methylisopropyl ketone are also determined by that reagent. Either monomethyl succinate or succinamic acid are determined as hydroxamic acids, page 315.

KETONES IN GENERAL

Ketones are determinable by oxidation with hypoiodite to iodoform. The method is not selective having been applied to acetone, butanone, 2-pentanone, 2-hexanone, 2-heptanone, and 2-octanone.[7] Acetaldehyde does not interfere.

A general reaction for aliphatic aldehydes and ketones is obtained with vanillin in alkaline solution.[8] The reaction hardly occurs at all with aliphatic compounds. For determination of ketones as phenylhydrazones[9] see page 278.

Procedure—*By iodine.* Prepare a solution of 200 grams of potassium iodide and 100 grams of iodine in 400 ml. of water. Mix, with cooling, 20 ml. of this solution, 6.6 ml. of 20% sodium hydroxide solution, and 20 ml. of the sample solution. Shake for 5 minutes. Add 5% sodium thiosulfate until the color is removed. Extract with 20 ml. of chloroform. Wash the chloroform layer with an equal volume of water. Dehydrate the chloroform layer with anhydrous sodium sulfate and read the iodoform at 347 mμ.

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[4] M. Mouton and M. Masson, *Ann. pharm. franc.* 17, 72-4 (1959).

[5] Adolfo Leandro Montes, *Anal. asoc. quím. Argentina* 42, 30-7 (1954).

[6] A. S. Maslennikov, *Zhur. Anal. Khim.* 13, 599-602 (1958).

[7] S. A. Shchukarev, S. N. Andreev, and O. V. Sapozhnikova, *Zhur. Anal. Khim.* 9, 193-5 (1954).

[8] V. E. Levine and M. Taterka, *Anal. Chim. Acta* 15, 237-45 (1956).

[9] Kenneth J. Monty, *Anal. Chem.* 30, 1350-2 (1958).

By vanillin. To 1-2 ml. of sample solution add 1 ml. of 1% vanillin solution and 2 ml. of 40% sodium hydroxide solution. Heat at 100° for 10 minutes and cool. Read at 508 mμ.

ACETONE

Salicylaldehyde reacts with acetone (Vol. III, p. 292) to produce a color which is probably the result of a condensation of the reagent with a methylene group in the α position to an unsaturated group such as to the carbonyl in acetone.[10] A pure reagent is preferred over an ethanolic solution for reproducible results. A very large excess of sodium hydroxide should be avoided as the solubility of the salicylaldehyde decreases with the increase in alkali concentration.

All compounds having carbonyl groups interfere such as acetaldehyde, diacetyl, acetophenone, benzoylacetone, pyruvic acid, levulinic acid, ethyl acetoacetate, propionaldehyde, methyl ethyl ketone, diethyl ketone, cyclohexanone, methyl cyclohexanone, cyanoacetic acid, and acetoacetic acid. Bisulfite ions and formaldehyde at high concentrations inhibit color development to some extent.[11] The color is stable for several hours after a two-hour development period. Beer's law is followed. Amounts of the order of 0.0025 mg. are determinable in deproteinized plasma without distillation.[12]

Determination of acetone in blood as the 2,4-dinitrophenylhydrazone (Vol III, p. 293) is accomplished by distillation of acetone into the reagent in the presence of phosphoric acid. The hydrazone is extracted with carbon tetrachloride.[13] The extinction coefficient as the 2,4-dinitrophenylhydrazone is 1.79 at 425 mμ and 0.850 at 530 mμ. This is also applicable to acetone in expired air.[14] For those of other ketones and aldehydes see Table 16.

A related micro method is based on the isolation of acetone by diffusion from the sample on a strip of filter paper into 2,4-dinitrophenylhydrazine solution.[15] A reddish-brown color is developed in alcoholic alkali and follows Beer's law for 0.0005-0.008 mg. The diffusion apparatus is assembled so that the reagent is kept chilled while the filter paper with the sample is heated to speed the transfer of acetone from the paper to the tube containing the reagent.

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[10] Svante Berntsson, *Ibid.* **28,** 1337 (1956).

[11] T. Thomson, *J. Soc. Chem. Ind.* **65,** 121-4 (1946).

[12] A. D. Marenzi and E. S. Braegger, *Publs. inst. invest. microquim. Univ. nacl. litoral (Rosario, Arg.)* **17,** 140-57 (1953); *Anales farm. y bioquim. (Buenos Aires)* **21,** 49-65 (1954).

[13] Julia Z. Rutman, *J. Lab. Clin. Med.* **41,** 648-52 (1953).

[14] D. Glaubitt and J. G. Rausch-Strooman, *Clin. Med. Acta* **4,** 165-9 (1959).

[15] M. U. Tsao, G. H. Lowrey, and E. J. Graham, *Anal. Chem.* **31,** 311-4 (1959).

The acetone dinitrophenylhydrazone shows a sharp absorption maximum at 430 mμ and a broad maximum at 540 mμ, the latter reading being preferred since the reagent blank is higher at 430 mμ. Mineral acid is included in the reagent to suppress a high reagent blank while the ethanol is added to keep the hydrazone in solution. Acetaldehyde interferes and when present in large concentrations, the reading is taken immediately after color development and after a 30-minute interval at 37°. The absorbance due to acetone drops 5% under these conditions while that due to acetaldehyde drops 75%. Sodium fluoride, sodium azide, mercuric chloride, dinitrophenol, malonic acid, or citric acid at 1-millimole concentrations do not interfere with the acetone determination in blood.

Acetone in air is determined by the pink color resulting on absorption in hydroxylamine hydrochloride solution.[16] Other ketones, aldehydes, and some acids give the same color development.

The oxidation production of acetone and other methyl ketones with alkaline hypobromite solution produces a red color after treatment with pyridine, the result of a reaction between pyridine and bromoform.[17] The reaction is quantitative with acetone and also can be used to determine other methyl ketones but not methyl carbinols. Condensation of furfural with acetone gives difurfurylideneacetone which has a red to violet color in acid solution.[18]

As little as 0.001% of acetone is determined in methylmethacrylate monomer by conversion to iodoform for reading the near ultraviolet.[19] The reaction is applicable to acetone in general. For reading acetone and divinylacetylene in the ultraviolet in vinyl acetate see page 10. Acetone is also developed with *p*-hydroxybenzaldehyde, page 44.

Procedure—*By 2,4-dinitrophenylhydrazine. Blood.* To a distillation apparatus, add 2 ml. of 0.3% sodium bisulfite solution and 0.2 ml. of sample. Place 2 ml. of 0.1% 2,4-dinitrophenylhydrazine solution in 1:5 hydrochloric acid in the receiving flask. Add 5 ml. of 1:500 phosphoric acid to the distilling flask and distil gently for 5 minutes. Lower the receiving flask and distil for one additional minute. Rinse the condenser into the distillate with water and dilute the distillate to 15 ml. with water. Extract acetone with 10 ml. of carbon tetrachloride by mechanical shaking for 15 minutes. Wash the chloroform layer with two 25-ml. portions of water and one 5-ml. portion of 2% sodium hydroxide solution. Read at 420 mμ against a reagent blank.

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[16] V. N. Petrov, M. A. Buzhenko, and A. A. Korobtsov, *Izvest. Vysshykh Ucheb. Zavedenii, Khim. i Khim. Tekhnol.* **2,** No. 3, 394-8 (1959).

[17] Vaclav Sedivec, *Chem. Listy* **51,** 63-7 (1957).

[18] Henri Etienne, *Ind. chim. belge* **17,** 455-8 (1952).

[19] Y. Takayama and F. Tokiwa, *Bull. Chem. Soc. Japan* **31,** 369-72 (1958).

Alternatively,[20] hemolyze 5 ml. of oxalated blood with 30 ml. of water. Deproteinize with 5 ml. of 10% sodium tungstate and 5 ml. of 1:45 sulfuric acid. Dilute to 50 ml. and centrifuge after 15 minutes. Add 10 ml. of 0.46% potassium dichromate in 1:1.3 sulfuric acid and 15 ml. of water to 25 ml. of the centrifugate. Distil into water and dilute to 10 ml.

As reagent dissolve 100 mg. of 2,4-dinitrophenylhydrazine in carbonyl-free methanol, add 0.4 ml. of concentrated hydrochloric acid, and dilute to 100 ml. with methanol. Mix 1 ml. of this reagent with 1 ml. of the distillate. After 30 minutes at 20° add 5 ml. of 10% potassium hydroxide in 80% methanol. Read at 550 mμ.

Urine. Mix 25 ml. with 5 ml. of 1:3 sulfuric acid and 20 ml. of water. Treat as for blood from "Distil into water and dilute to 10 ml."

By hydroxylamine. Air. Add 0.3 ml. of 0.1% methyl orange solution to 100 ml. of 0.2% hydroxylamine hydrochloride solution as reagent. Place 12.5 ml. of this in appropriate absorbers. Pass a known volume of the gas through the reagent until a deep pink develops. This corresponds to absorption of 2-2.5 mg. of acetone. Dilute the contents of the absorbers to 25 ml. and read.

Microdetermination—Prepare glass diffusion rods 100 mm. long with a 3-mm. diameter with a flattened segment 20 mm. from the lower end to which a stainless steel wire can be fastened. Attach a part of the wire so that it forms a loop around the rod as a guide for sliding the filter paper with the sample into the photometer tube, Figure 12. Cut 8 × 16 mm. filter paper strips to form a collar around the diffusion tube by notching the ends so that they may be hooked together. To keep the filter paper from falling off, flatten the end of the glass rod so that the paper may be slipped past but cannot slip down. Set the upper end of the diffusion rod into a one-hole No. 0 rubber stopper which fits into the tube.

Prepare a diffusion rack as shown in Figure 12. Start with a desiccator D of 21-cm. diameter which is used as an ice bath and a support for the diffusion rack, which holds the tubes. Prepare three flat stainless steel rings fastened together with four vertical rods, the bottom ring which

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[20] H. Böhme and O. Winkler, *Hoppe-Seyler's Z. physiol. Chem.* **296**, 274-9 (1954).

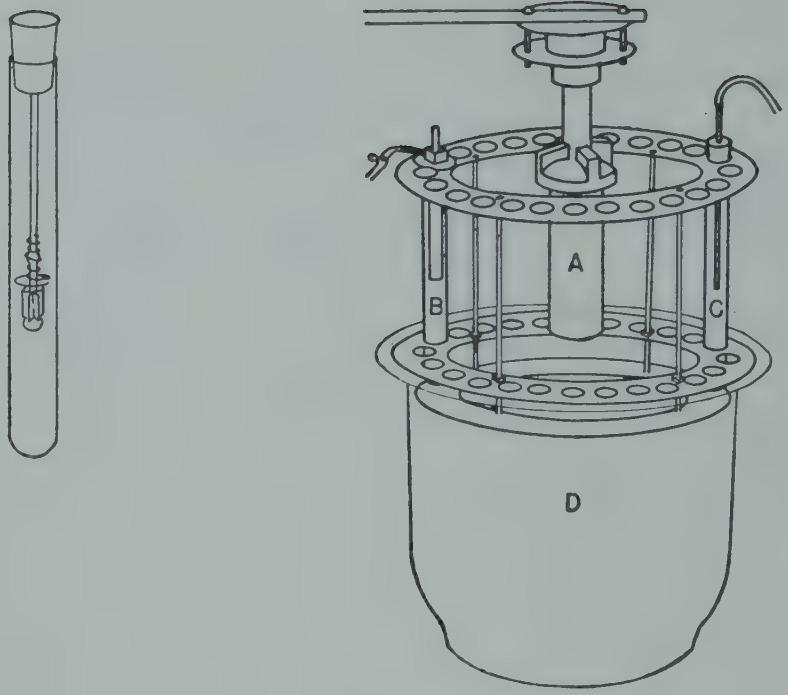

Fig. 12

Equipment for microdetermination of acetone
Left: Diffusion rod with filter paper strip; *Right:* Diffusion rack and ice bath.

contains no holes so as to support the tubes, the center ring which rests on the rim of the desiccator and has holes for the tubes, and the top ring which also has holes to support the tubes. Space the rings so that the tubes rest securely in the rack with 10.5 cm. of the tube above the center ring and 2 cm. below it.

To provide heat for the filter paper, suspend a heating element in the center of the rack. Place a thermoswitch B in a hole in the rack to maintain the temperature reading from the thermistor-type thermometer C in the tube at 37-39° when the top ring has its center area covered with asbestos shields. Operate the thermoswitch with a variable transformer which supplies the current to the heating element at approximately 40 volts. Provide a metal shield 18 cm. high, the inside surface of which is highly polished, to encircle the tubes, act as a reflector, and facilitate even heating. Attach a stainless steel disk of a diameter small enough to pass through the opening of the top ring and yet large enough to rest on the center ring to support an asbestos disk to insulate the ice bath in the desiccator from heating. Add an asbestos roof to the top of the diffusion rack to cut down air convection currents.

As 2,4-dinitrophenylhydrazine reagent, mix 50 ml. of 95% ethanol and 0.25 ml. of concentrated sulfuric acid and heat a 10 ml. portion with 10 mg. of 2,4-dinitrophenylhydrazine to 50° with constant shaking to dissolve. Centrifuge. Prepare fresh for each determination. Pipet 0.15 ml. of the reagent into the bottom of the tube. Put the notched paper on the diffusion rod and place the rod end way into the tube so that the rod can be quickly slid into place and the tube stoppered with a minimum evaporation of acetone. Pipet a 0.01-0.02 ml. sample onto the paper and immediately stopper. Place the tube in the diffusion rack which has been thermally equilibrated at 37-39° and fill the desiccator with ice and water. Allow the diffusion to proceed for 1.5 hours. Remove the tubes and let stand for 30 minutes at room temperature. Add 0.3 ml. of 36% sodium hydroxide solution, mix, and let stand exactly 30 seconds. Add 3 ml. of 50% ethanol and read against a reagent blank at 540 mμ at a fixed time interval after addition of the ethanol.

By salicylaldehyde. To a sample containing 0.009-0.016 mg. of acetone, add 2 ml. of 42.5% sodium hydroxide solution. Dilute with water to 25 ml. and add 0.6 ml. of pure salicylaldehyde. Shake, add 20 ml. of 42.5% sodium hydroxide solution, and dilute to 50 ml. with water. Let stand 2 hours for color development and read at 474 mμ against a reagent blank.

Air.[21] Aerate through 20 ml. of 1% sodium bisulfite solution at 100 ml. per minute. Add 1 ml. of salicylaldehyde to a 5-ml. sample containing 10-50 mg. of acetone. Heat in boiling water and cool. Dilute to 15 ml. and let stand for 15-25 minutes. Read at 474 mμ.

By hypobromite and pyridine. To 5 ml. of solution prepared by mixing 10 ml. of 40% sodium hydroxide solution with 100 ml. of saturated bromine water, add a 5-ml. sample solution. Stir and let stand for 5-10 minutes to oxidize the acetone. Destroy the excess sodium bromate with 1 ml. of 10% sodium thiosulfate. Add 10 ml. of pyridine and heat for 18-20 minutes at 50 ± 1°. Cool in ice, dilute to 25 ml. and read.

By furfural. The distilling apparatus should have ground-glass joints. Place 5 ml. of sample containing 0.005-0.05 mg. of acetone in the distilling flask. Add 5 ml. of 40% potassium cyanide solution. Place in the receiver 0.2 ml. of 5% furfural in methanol and 0.5 ml. of 10% sodium hydroxide solution. Distil over a volume of 2.5 ml. After standing for 30 minutes add 15 ml. of 1:1.5 sulfuric acid to the receiver to develop the color. Read at 425 mμ.

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[21] T. Fukuyama, T. Sato, and A. Watanabe, *Bull. Inst. Public Health (Tokyo)* **5**, No. 1, 2-7 (1956).

As iodoform. Methylmethacrylate. Mix 7 ml. of 20% iodine solution with 2 ml. of 25% sodium hydroxide solution. Shake until no longer orange-yellow and add more iodine solution until it no longer reacts. Add 1 ml. of methylmethacrylate containing no more than 0.4 mg. of acetone to the hypoiodite solution. Shake for 1 minute at room temperature and add iodine solution dropwise until the color is restored. Decolorize with 5% aqueous sodium thiosulfate solution. Extract the iodoform by shaking with 14 ml. of chloroform. Wash the chloroform layer by shaking with an equal volume of water. Dehydrate the chloroform layer with anhydrous sodium sulfate and read at 347 mμ.

ACETYLACETONE

A determination of acetylacetone is based on its reaction with ferric ion to produce a soluble red ferric salt.[22] The optical density read depends on the concentration of reagent. The method is also applicable to determination of β-(*p*-toluidino) propenyl ketone, a Schiff's base of acetylacetone, by hydrolysis to acetylacetone.

An alternative is determination as the copper complex.[23] The sample must be preadjusted to pH 5.2-6.

Procedure—*By ferric alum.* To 5 ml. of 6.5% ferric alum solution in 1:350 sulfuric acid, add a 1-15 ml. sample of acetylacetone solution in 1:350 sulfuric acid. Dilute to 20 ml. with 1:350 sulfuric acid, let the color develop for 15 minutes, and read at 480 mμ against a reagent blank.

By cupric acetate. Mix a sample containing 0.3-0.5 gram of acetylacetone with 25 ml. of 5% cupric acetate monohydrate solution and 25 ml. of 20% sodium acetate trihydrate solution. Dilute to 250 ml. and shake occasionally for 5 minutes. Shake with 20 and 20 ml. of carbon tetrachloride which has been saturated with the copper complex of acetylacetone. This should remove extraneous color; if not, extract with further portions. Extract the copper complex with four successive 20-ml. portions of chloroform. Combine these extracts, dilute to 100 ml., and read at 650 mμ.

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[22] T. G. Bonner and M. Thorne, *Analyst* **79**, 759-61 (1954).
[23] R. J. Starkey, *Ibid.* **84**, 517-9 (1959).

METHYL ISOPROPENYL KETONE

The determination of combined methyl isopropenyl ketone in butadiene-methyl isopropenyl ketone copolymers and butadiene-methyl isopropenyl-acrylonitrile tripolymers is by direct reading in chloroform at 290 mμ.[24] The absorptivity of the methyl isopropenyl ketone polymer is 0.57 as compared with about 0.01 for polybutadiene and butadiene-acrylonitrile. The assumption is made that the absorptivity of the ketone in copolymers is the same as in the polymer. Phenyl-2-naphthylamine interferes. Therefore so far as possible antioxidants should be absent.

Procedure—*Butadiene-methyl isopropenyl ketone copolymers and butadiene-methyl isopropenyl-acrylonitrile tripolymers.* Dissolve a 0.6-gram raw sample in 100 ml. of 1:4 methanol-chloroform by mechanical shaking. Add 300 ml. of methanol to a high-speed blender, start the blender at a medium speed, and slowly add the polymer solution. Filter the precipitated polymer with suction, scraping all polymer from the blades and container of the blender. Wash with two 25-ml. portions of methanol. The purified polymer should be white at this stage. Dissolve in 80 ml. of chloroform. Transparent gel may be present.

Transfer to a Seitz filter apparatus which has been fitted with a prepared filter disk and operates under a nitrogen pressure of 60 pounds per square inch. Filter and dilute the filtrate to 100 ml. with chloroform. Weigh an aluminum dish and evaporate the chloroform from a 25-ml. aliquot at 50°. Reweigh. Adjust the sample concentration to 2-3 grams per liter. Read at 290 mμ against chloroform. Calculate the per cent of methyl isopropenyl ketone as follows:

$$\%\ \text{methyl isopropenyl ketone} = 178.5\, a_{\text{total}} - 1.8$$

where

$$a_{\text{total}} = \frac{\text{absorbance read}}{\text{concentration in grams per liter}}.$$

BENZYLIDENE ACETONE

A method for benzylidene acetone is based on its yellow color with sulfuric acid.[25]

Procedure—*Ointment.* Repeatedly triturate a 0.15-1.5-gram sample of 5% ointment with 5-ml. portions of 1:1 sulfuric acid until a colorless extract is obtained. Dilute the combined extracts to 50 ml. with 1:1 sulfuric acid and read at 460 mμ.

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[24] J. J. Pepe, I. Kniel and M. Czuha, Jr., *Anal. Chem.* 27, 755-8 (1955).

[25] R. Dolique and H. Pages, *Trav. soc. pharm. Montpelier* 15, No. 1, 51-7 (1955).

ACETYLMETHYLCARBINOL, ACETOIN

Acetylmethylcarbinol (Cf. Vol. III, pp. 297-8) is determined chemically by oxidation with strong alkali in the presence of air to diacetyl followed by determination with α-naphthol and creatine.[26] Distillation to separate from interfering substances is sometimes necessary.

Procedure—*Citrus juices.* Prepare an oxidizing reagent by dissolving 0.3 gram of creatine in 100 ml. of 40% potassium hydroxide solution. Mix a 20-ml. sample containing 5 grams of powdered pumice stone with 4 ml. of oxidizing solution and 10 ml. of 5% α-naphthol in 95% ethanol. Stopper and shake vigorously for 1 minute. Let stand for 10 minutes, centrifuge for 10 minutes, and decant. Read the red color of the supernatant liquid against a control.

Alternatively, distil 20 ml. from a 300-ml. sample. Take a 10-ml. aliquot without peel oil which would cause cloudiness. Add 2 ml. of the potassium hydroxide reagent and 5 ml. of 5% α-naphthol in 95% ethanol. Stopper, shake vigorously for 15 seconds, and let stand for 10 minutes. Shake for 15 seconds and read the red color against a reagent blank.

2,3-BUTANEDIONE, DIACETYL

Diphenyltetramine hydrochloride as a reagent for diacetyl produces a yellow diphenylquinoxaline.[27] The color when heated in concentrated sulfuric acid is also applicable.[28] In the presence of creatine and α-naphthol (Vol. III, p. 300), diacetyl yields a red color.

Procedure—*By diphenyltetramine. Butter.* Steam-distil a 50-gram sample at 110° until 15 ml. of distillate is obtained. Add 0.75 ml. of a 2.5% diphenyltetramine hydrochloride solution and 0.75 ml. of concentrated hydrochloric acid. Read the yellow color of the diphenylquinoxaline solution or compare with 1% potassium dichromate solution.

In concentrated sulfuric acid. Extract a solution containing not more than 0.09 mg. of diacetyl thrice with appropriate volumes of chloroform.

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[26] E. C. Hill, F. W. Wenzel, and A. Barreto, *Food Technol.* 8, 168-71 (1954).

[27] Manlio Brogioni, *Boll. lab. chim. provinciali* 5, 50-3 (1954).

[28] G. Lotti, C. Galloppini, and L. Carloni, *Ann. fac. agrar. univ. Pisa* 19, 39-50 (1958).

To the combined extracts add 5 ml. of 0.2% solution of resorcinol in chloroform. Add 20 ml. of concentrated sulfuric acid and shake thoroughly. Let stand for two minutes and separate the sulfuric acid layer. Heat it at 100° for 20 minutes and cool. Read at 542 mμ.

PHENYLACETONE

Phenylacetone is an intermediate in production of amphetamine, α-methylphenethylamine. It is read directly in ethanol. Selection of 290 mμ rather than the peak at 285 mμ decreases the interference.[29]

Procedure—Dilute a 10 ml. sample of amphetamine solution, containing 0.01-0.1 gram of phenylacetone, to 100 ml. with 95% ethanol. Read at 290 mμ against an ethanol blank.

CARVONE; 6,8(9)-p-METHADIENE-2-ONE

Using ethyl 3,5-dinitrobenzoate in methanol as a reagent, carvone can be determined in the range of 1-10 mg. per ml.[30] Beer's law is followed. The Girard-T hydrazone of carvone is read at 272.5 mμ.[31]

Procedure—To a 4-ml. sample containing a minimum of 1 mg. of carvone per ml. add 5 ml. of 2% ethyl 3,5-dinitrobenzoate solution in methanol. Add 1 ml. of 2.8% sodium hydroxide solution and read exactly 3.5 minutes later at 537.5 mμ and 20° against a reagent blank.

α-, β-, γ-THUJAPLICINS; 2-HYDROXY-3-, 4-, AND 5-ISOPROPYL-2,4, 5-CYCLOHEPTATRIEN-1-ONE

When α-, β- and γ-thujaplicins, fungicides occurring in the heartwood of certain trees, are treated with the ferric ion, a red chelate forms which is soluble in organic solvents.[32] Solvent extraction is preferred over steam distillation for isolating the thujaplicin as being more rapid. Although diethyl ether is a good solvent for the thujaplicins, it cannot be used with wood samples since it does not extract the thujaplicins from the ether-insoluble membrane substances. Water is the best solvent followed by an extraction with hexane to remove thujaplicins from the pyrocatechol-type polyphenols which are extracted with hot water and would form brown precipitates with ferric ion. The hexane is diluted with chloroform since ferric chelates are only slightly soluble in this solvent.

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[29] Stanley K. Freeman, *J. Am. Pharm. Assoc.* **42**, 621-2 (1953).

[30] D. H. E. Tattje, *Pharm. Weekblad* **91**, 733-6 (1956); F. H. L. van Os and C. Scholtens, *Riechstoffe u. Aromen* **8**, 265-8 (1958).

[31] J. B. Steulake and W. D. Williams, *J. Pharm. and Pharmacol.* **9**, 900-7 (1957).

[32] H. MacLean and J. A. Gardner, *Anal. Chem.* **28**, 509-12 (1956).

Nonphenolic extracts soluble in organic solvents such as thujic acid and its methyl esters do not react with the ferric ion. An excess of ferric iron or the presence of acid converts the red chelate to a green water-soluble complex. Above pH 4.9 difficulties are encountered with the formation of stable emulsions and at a pH below 4.3 the color formation from the α-isomer increases. Therefore a pH of 4.3-4.9 is chosen for the method.

Beer's law is not followed for the isomers. It is possible, however, to calculate the percentages of β- and γ-isomer in a mixture since a 1:1 mixture of the two gives a color response halfway between that of pure β- or pure γ-isomer. The position of the maximum in the 425 mμ region for the ferric chelates of thujaplicins relative to those of the pure β- and γ-isomers, assuming the α-isomer to be absent or present in small amounts, is a rough measure of the relative occurrence of the two isomers.

In the preparation of the calibration curve with mixed isomers, the percentages of the isomers in the sample must be considered if the calibration curve is to be valid. However the ratio is usually not widely varied and in routine analyses the same calibration curve may be used. Direct sunlight causes color fading.

Procedure—*Wood.* Heat a 2-gram, 40-mesh sample for 1 hour at 100° with 100 ml. of water in a flask fitted with an air condenser. Decant the hot extract through a fritted glass crucible. Repeat the extraction of thujaplicins using a 50-ml. portion of water and heating for 1 hour, followed with a 25-ml. portion of water and heating for 30 minutes. Wash the residue with 25 ml. of hot water. Dilute the combined cooled extracts to 200 ml. with water and extract the thujaplicins from a 5-ml. aliquot with two 5-ml. portions of *n*-hexane, shaking for 1 minute each.

Prepare a reagent by dissolving equimolar quantities of sodium acetate and ferric chloride in sufficient water to make the final solution 1% in iron content. This reagent should have a pH of 4.3. Add 5 ml. of this reagent and 10 ml. of chloroform to the combined hexane extracts. Shake for 5 minutes and filter the organic layer. Wash the aqueous layer with chloroform, add the chloroform washings to the filter, and dilute the filtrate to 25 ml. Read at 425 mμ against 2:3 hexane-chloroform.

Crystalline or oily material. Use a *n*-hexane solution of approximately 5 mg. per 100 ml. and a 10-ml. aliquot. Start at "Prepare a reagent. . . ."

ALLETHROLONE, 2-ALLYL-4-HYDROXY-3-METHYL-2-CYCLOPENTEN-1-ONE

Allethrolone is read directly in the ultraviolet in ethanol solution.[33] Beer's law is followed.

Procedure—Prepare a solution containing approximately 0.1 gram of allethrolone per liter in SD30 ethanol. Dilute a 4- or 6-ml. aliquot to 100 ml. and read at 231 mμ.

CAMPHOR

The color reactions of camphor with aldehydes (Vol. III, p. 301) have been applied with *p*-dimethylaminobenzaldehyde in sulfuric acid.[34] The yellow color conforms to Beer's law and checks closely with the official NF method.

Procedure—As reagent dissolve 0.125 gram of *p*-dimethylaminobenzaldehyde in 65 ml. of concentrated sulfuric acid and 35 ml. of water. Prepare fresh daily. Take 1 ml. of sample solution in chloroform containing 0.6-7.5 mg. of camphor. Add 7 ml. of the prepared reagent and mix. Let stand for 2 hours to develop the maximum color and read at 460 mμ.

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[33] Stanley K. Freeman, *Ibid.* **25**, 645-6 (1953).

[34] Martin I. Blake and Lola V. Hopkins, *Drug Standards* **27**, 39-40 (1959).

CHAPTER 13

UNSUBSTITUTED MONOBASIC ALIPHATIC ACIDS AND THEIR ANHYDRIDES, ESTERS, AND LACTONES

When acids are converted to their alkaline salts the wave length of maximum absorbancy is increased and the intensity of absorbancy at the peak is also increased. For more detail see page 77. Many esters of monobasic aliphatic acids are determinable as hydroxamic acids, page 315. They include ethyl acetate, methyl methoxyacetate, ethyl chloroacetate, ethyl-*l*-β-phenylacetate, methyl propionate, ethyl lactate, ethyl-β-ethoxypropionate, and methyl butyrate.

Esters, Anhydrides, and Lactones

The formation of hydroxamic acids which then react with ferric ion is a general reaction. For correlation therefore it is presented here as including esters of substituted monobasic acids, polybasic acids, and cyclic acids although those structures are classified as belonging in later chapters. It also includes their acid chlorides and anhydrides. The general reaction in which Y is an ester group, halogen, or the half-oxygen of an anhydride is $RCOOY + NH_2OH \rightarrow RC(O)NHOH + HY$. The hydroxamic acids produced form highly colored chelate compounds with ferric ion.[1]

Under the specified alkaline conditions esters react. In alkaline solution anhydrides react to form one mole of hydroxamic acid and one mole of the acid of the anhydride as its salt but the reaction is only 65% complete with 10 minutes of refluxing. Lactones react completely. The specified conditions are such that acids, most amides, and nitriles do not interfere. The colored chelates of copper, nickel, and vanadium interfere. Ions which complex with iron such as chloride, tartrate, acetic acid, and water can affect the final intensity of color.

In acid solution the ratio of the hydroxamic acid to ferric ion is 1:1. Excess hydroxylamine reduces ferric ion irreversibly with a maximum reaction rate at pH 1.4. Increase of pH shifts the maximum absorption toward shorter wave lengths and increases the extinction. Entering the alkaline range results in precipitation of the salt of one ferric ion with three hydroxamic acid ions. Further increase in the pH causes formation of a ferric-trihydroxamic acid ion. Beer's law holds over a wide range of concentration of ferric ion at pH 9.2.[2] The ferric complexes of most aliphatic hydroxamic acids have their absorption maxima at 530 mμ and the complexes of aromatic esters at 550-560 mμ. Complexes of esters of acids containing conjugated double bonds or more than one carboxyl group have different wave lengths. Esters of resin acids do not react. The substituted groups effect the rate of hydroxamic acid formation.[3]

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[1] R. F. Goddu, N. F. LeBlanc, and C. M. Wright, *Anal. Chem.* 27, 1251-5 (1955); E. Bayer and K. H. Reuther, *Chem. Ber.* 89, 2541-6 (1956).

Esters require 2-120 minutes for maximum color formation, showing correlation between structure and rate of hydroxamic acid formation. Bulky and electron-donating groups inhibit hydroxamic acid formation by substituted benzoic acid esters. Replacement of the hydrogen atoms of formamide by a methyl or phenyl group results in a decreased rate of color formation. The speed of reaction of *ortho*-substituted and *para*-substituted methyl benzoate esters varies inversely with the electron-releasing strengths of these substituents. There is also steric hindrance by bulky groups. Monocarboxylic acid amides which have no electron-withdrawing substituents react very slowly with the hydroxylamine reagent.

The applicability of the hydroxamic acids with ferric ion includes ketones,[4] diacylamides,[5] amides and nitriles,[6] and even volatile esters.[7] Details of determination of acetic anhydride in the presence of ketene in the air appears on page 295.

Procedure—*As hydroxamic acids by ferric ion.* In the series of determinations which follow, with one exception 2B ethanol is called for. Isopropanol is equally satisfactory. Benzene may be used. Purified dioxane, methylene chloride-ethanol mixture, petroleum ether, or purified diethyl ether are satisfactory. Water is applicable with some reduction in sensitivity.

Esters in alkaline solution. Reflux 12.5 grams of hydroxylamine hydrochloride with 100 ml. of methanol for a few minutes. Similarly reflux 12.5 grams of sodium hydroxide with 100 ml. of methanol. Mix equal volumes of the two solutions as the reagent and filter off sodium chloride.

To prepare the ferric perchlorate, dissolve 5 grams of ferric perchlorate in 10 ml. of 70% perchloric acid and 10 ml. of water. Dilute to 100 ml. with anhydrous ethanol, cooling as the ethanol is being added. Alternatively, add 10 ml. of 70% perchloric acid to 0.8 grams of iron wire and heat cautiously at low temperatures until the iron dissolves. Cool and take up with 10 ml. of water. Dilute cautiously to 100 ml. with anhydrous 2B ethanol, cooling as the ethanol is added.

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[2] Gunnar Aksnes, *Acta Chem. Scand.* **11**, 710-6 (1957).

[3] V. Goldenberg and P. E. Spoerri, *Anal. Chem.* **30**, 1327-30 (1958).

[4] W. M. Diggle and J. C. Gage, *Analyst* **78**, 473-9 (1953).

[5] J. C. Polya and P. L. Tardew, *Anal. Chem.* **23**, 1036-7 (1951).

[6] S. Saloway and A. Lipschitz, *Ibid.* **24**, 898-900 (1952).

[7] Adrienne R. Thompson, *Australian J. Sci. Research* **3A**, 128-35 (1950).

As a reagent add 12 ml. of 70% perchloric acid to 40 ml. of the ferric perchlorate solution and dilute to 1 liter with anhydrous 2B ethanol, adding the 2B ethanol in 50-100 ml. increments and cooling after each addition until the perchloric acid is diluted to about 10% of its original concentration.

Dissolve the sample in anhydrous 2B ethanol so that the concentration of the ester is 0.01-0.001 *M*. To a 5-ml. aliquot, or a smaller aliquot diluted to 5 ml. with ethanol for more concentrated samples, add 3 ml. of reagent. Reflux for 5 minutes, cool to room temperature, and take up with

TABLE 18. WAVE LENGTH FOR MAXIMUM ABSORPTIVITY AND MOLAR ABSORPTIVITY OF FERRIC HYDROXAMATES

Esters	*Wave length,* mμ	*Molar absorptivity,* $\times 10^3$
Ethyl formate	520	1.06
Ethyl acetate	530	1.10
n-Butyl acetate	530	1.06
n-Amyl acetate	530	1.05
Phenyl acetate (impure)	530	0.99
Triacetin	530	3.33
Ethyl propionate	530	1.02
γ-Butyrolacetone	530	1.11
Methyl *n*-butyrate	530	1.06
n-Butyl *n*-butyrate	530	1.05
n-Amyl *n*-butyrate	530	0.91
Dimethyl malonate	520	1.72
Dimethyl maleate	520	1.53
Dimethyl adipate	530	2.04
Pentaerythritol tetracaproate	530	3.89
Methyl oleate	530	1.00
Methyl benzoate	550	1.13
n-Butyl benzoate	550	1.08
Benzyl benzoate	550	1.13
Methyl *p*-toluate	550	0.94
Dimethyl *o*-phthalate	540	1.48
Dimethyl isophthalate	540	2.42
Dimethyl terephthalate	540	2.37

ferric perchlorate reagent. Dilute to 50 ml. with the reagent and shake to dissolve the precipitated ferric hydroxide. Read after several minutes against a reagent blank at the wave length of maximum absorbance of the ester being analyzed. Appropriate wave lengths are shown in Table 18.

Esters, anhydride present. Determine the amount of anhydride by neutral hydrolysis in the presence of esters. Determine the total of ester and anhydride by alkaline hydrolysis. Subtract the absorbance due to the anhydride under neutral hydrolysis conditions to get the amount of ester present.

Lactones in the presence of esters. Proceed as for anhydrides but with alcohols, ethers, or hydrocarbons as solvents. The ester is not reacted in this technic.

Anhydrides in the presence of esters. To prepare the hydroxylamine reagent, reflux 12.5 grams of hydroxylamine hydrochloride with 100 ml. of methanol for a few minutes. Similarly reflux 12.5 grams of sodium hydroxide with 100 ml. of methanol. Neutralize a portion of the hydroxylamine hydrochloride solution to the phenolphthalein end point with the sodium hydroxide solution, and filter off the sodium chloride.

Dissolve the anhydride in dry benzene so that its concentration is 0.01-0.001 *M*. Follow the procedure for esters substituting a 10-minute refluxing period for the 5-minute period in the procedure. The ester is not reacted in neutral solution.

Ester Groups in Lipids

The hydroxamic acid reaction is applicable to ester groups, whether of simple or poly alcohols. The acid is then read in the presence of ferric perchlorate.[8]

Procedure—As a stock reagent dissolve 5 grams of ferric perchlorate in 10 ml. of 70% perchloric acid and 10 ml. of water. Dilute to 100 ml. with cold absolute ethanol and store in a refrigerator. For use dilute 4 ml. of the stock reagent and 3 ml. of 70% perchloric acid to 100 ml. with cold absolute ethanol.

As hydroxylamine reagent first dissolve 2 grams of hydroxylamine hydrochloride in 2.5 ml. of water and dilute to 50 ml. with absolute ethanol. Dissolve 4 grams of sodium hydroxide in 2.5 ml. of water and dilute to 50 ml. with absolute ethanol. Mix equal volumes of these two solutions and centrifuge to separate the salt formed.

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[8] F. Snyder and N. Stephens, *Biochim. et Biophys. Acta* **34**, 244-5 (1959).

Add 1 ml. of fresh hydroxylamine reagent to the dry sample of lipid. Incubate at 65° for 2 minutes and let cool for 5 minutes. Add 2.5 ml. of fresh ferric perchlorate reagent. Read after 30 minutes at 530 mμ against a reagent blank. The color is stable for more than an hour.

FORMIC ACID

Conditions affect the formation of hydroxamic acids for estimation by the color with ferric ion. At a low pH, formic acid forms the hydroxamic acid by reaction with hydroxylamine in methanol. The iron salt of this particular hydroxamic acid is violet.[9] The method is also applicable to formic acid esters.[10] Other aliphatic acids give similar reactions (Cf. Vol. III, p. 306).

Procedure—To prepare the ferric perchlorate reagent, dissolve 0.8 gram of iron in 10 ml. of 70% perchloric acid and dilute with 10 ml. of water. Dilute to 100 ml. with 95% ethanol. Acidify a 4-ml. portion with 1.2 ml. of 70% perchloric acid and dilute to 100 ml. with 95% ethanol. Acidify 0.5 ml. of an aqueous solution of formic acid with concentrated hydrochloric acid to a pH close to 0. Add 1 ml. of methanol and after 15 minutes add 1 ml. of 10% hydroxylamine solution in methanol and 0.5 ml. of 0.4% sodium hydroxide solution. Let stand for 15 minutes and add 10 ml. of ferric perchlorate solution. Read at 500 mμ.

ACETIC ACID

When acetate ion is treated with iodine, lanthanum ion, and ammonia (Vol. III, pp. 306-7) the bluish green to green of iodine sorbed on the lanthanum acetate is read at 660 mμ.[11] This is applicable to estimation of 0.25-1 mg. in urine. The same reaction with praseodymium or neodymium is applicable to 7-10 mg. of acetate ion per ml. at pH 6.5 or 4.8 mg. per ml. at pH 6.[12]

Acetic acid is very stable to oxidation by dichromate in 50% sulfuric acid whereas practically all possibly interfering substances are oxidized.

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[9] Maurice Pesez, *Ann. pharm. franç.* **15,** 173-6 (1957).

[10] E. E. Vonesch and O. A. Guagnini, *Microchim. Acta* **1958,** 1-8.

[11] Koichiro Oono, *J. Biochem (Tokyo)* **39,** 133-5 (1952).

[12] K. Kimura, N. Ikeda, and M. Nomura, *Bull. Chem. Soc. Japan* **26,** 119-22 (1953).

In 60% sulfuric acid in the presence of silver ion the acetate ion is oxidized by dichromate. Therefore 0.1-1 mg. of acetic acid is determined by the dichromate ion reduced under the latter conditions.[13]

Procedure—*By lanthanum. Urine.* To a 25-ml. sample add 4 ml. of 1:3 sulfuric acid and 15 ml. of 10% sodium tungstate solution, and mix. Add 8 grams of magnesium sulfate heptahydrate and steam-distil until 200 ml. of distillate is collected. To the distillate add 2 ml. of 1:3 sulfuric acid, 50 grams of magnesium sulfate heptahydrate, and 10 ml. of 10% mercuric sulfate solution in 1:6 sulfuric acid. Repeat the steam distillation collecting 190-200 ml. Add 10 ml. of a saturated barium hydroxide solution to the distillate and evaporate to approximately 5 ml. at 100°. Pass carbon dioxide gas through the mixture to convert barium hydroxide to barium carbonate. Adjust the pH of the filtrate to 9 with a saturated barium hydroxide solution. Dilute to 15 ml. and filter.

To a 1-ml. aliquot containing 0.1-0.4 mg. of acetic acid add 1 ml. of 0.2538% iodine solution, 0.5 ml. of 2.8% lanthanum nitrate solution, and 0.5 ml. of 1:99 ammonium hydroxide. Mix and heat at 100° for 5 minutes. Cool and dilute to 6 ml. Read at 660 mμ.

By dichromate oxidation. Urine. Make a 10-ml. sample alkaline by addition of solid sodium hydroxide. Add 0.5 gram of copper sulfate pentahydrate, shake well, and let stand for at least 12 hours in a refrigerator. Centrifuge, take a 5-ml. aliquot, and dilute with 5 ml. of water. Add 2.5 ml. of a saturated copper sulfate solution and 4 ml. of 15% calcium hydroxide suspension. Filter and evaporate over a boiling saturated calcium chloride solution. Take up the residue in 15 ml. of methanol and 3 ml. of concentrated sulfuric acid. Distil, collecting 8 ml. of distillate in 4 ml. of methanol. Repeat the distillation 3 times, collecting the last fraction in 4 ml. of 1.25% sodium hydroxide solution to saponify the methyl ester. Evaporate to dryness and add 10 ml. of 1:1 sulfuric acid. Heat to 50° and add 1% potassium dichromate solution dropwise, until the yellow color persists fairly strongly. The acetate is not oxidized. Cool and read the yellow in an aliquot of the solution at 440 mμ. Dilute 5 ml. of the oxidized solution to 10 ml. with 3:1 sulfuric acid, the final concentration of sulfuric acid then being over 60%. Add saturated aqueous silver sulfate solution dropwise until reaction ceases. The acetate is now oxidized. Read the yellow color at 440 mμ and calculate the acetate from the amount of dichromate reduced to chromic ion.

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[13] Alberto Fonnesu, *Biochem. Z.* 324, 512-6 (1953).

ACETIC ANHYDRIDE

In the microdetermination of acetic anhydride, sodium nitrate, sodium acetate, acetone, and 3-amino-4-methoxy-6-benzamidotoluene combine as a reagent. For larger quantities, dimethyl aniline is substituted for 3-amino-4-methoxy-6-benzamidotoluene.[14]

Acetic anhydride is also determined by the general form of the reaction to form a mole of hydroxamic acid as discussed under the general reaction.[15] For determination in air the value is the sum of acetic anhydride and ketene after absorption in alkaline hydroxylamine. For separate determination of the ketene first absorb the anhydride in toluene.

Procedure—*Micro.* Prepare a reagent containing 80 ml. of 8.2% sodium acetate solution, 100 ml. of acetone, and 0.2 gram of 3-amino-4-methoxy-6-benzamidotoluene. Warm to dissolve, cool, and add 20 ml. of 8.5% sodium nitrate solution. To 5 ml. of the reagent, add 0.5-2 ml. of sample solution containing 0.005-0.1 mg. of acetic anhydride in acetone. Mix and after 5 minutes dilute with 0.05% 1-amino-8-naphthol-2,4-disulfonic acid in 1% sodium carbonate solution to 25 ml. Read with a yellow-green filter.

Macro. To prepare the reagent, mix 20 ml. of 8.5% sodium nitrate solution, 80 ml. of 8.2% sodium acetate solution, and 100 ml. of acetone containing 0.2 ml. of dimethyl aniline. To 10 ml. of reagent, add 0.5-2 ml. of sample containing 0.2-2 mg. of acetic anhydride in acetone. Dilute to 25 ml. with the reagent and read after 10-15 minutes against a reagent blank using a violet filter.

KETENE

Ketene, a colorless, highly toxic gas, reacts with hydroxylamine to give acetohydroxamic acid the ferric complex of which is purple.[16] Carboxylic acids do not interfere but acid chlorides, anhydrides, and esters should be absent. To eliminate the interference of acetic anhydride which would probably be present in air samples containing ketene, the air sample is first passed through an absorber containing 20 ml. of toluene which absorbs approximately 74.2% of the acetic anhydride and then passed through an absorber containing alkaline hydroxylamine hydrochloride solution.

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[14] H. F. Liddell and B. Saville, *Chem. & Ind. (London)* 1957, 493-4.

[15] W. M. Diggle and J. C. Gage, *Analyst* 78, 473-9 (1953).

[16] W. M. Diggle and J. C. Gage, *Analyst* 78, 473-9 (1953).

The acetic anhydride in the toluene is extracted with alkaline hydroxylamine solution and the colors in both absorbers are developed as the ferric complex of the hydroxamic acid. The amount of acetic anhydride contributing to the reading in the hydroxylamine hydrochloride absorber is subtracted to determine the amount of ketene present. If the ratio of acetic anhydride to ketene is above 20:1 this procedure would not be valid.

Procedure—The apparatus required is shown in Figure 13. Add 20 ml. of toluene to the first absorber H and 5 ml. of 20% hydroxylamine hydrochloride solution and 5 ml. of 20% sodium hydroxide solution to the second absorber H. Open the stopcock G and draw air through the absorbers for 15 minutes at the rate of 6 liters per minute by means of the vacuum pump J. Control the rate of flow with the screw clip K and measure the rate with the rotometer L.

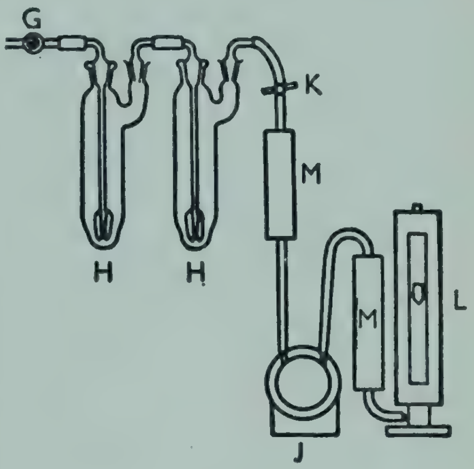

FIG. 13

Absorber for acetic anhydride in air samples containing ketene

G = the stopcock to let in air sample; H = two absorbers; J = vacuum pump; K = screw clip to control rate of sampling; L = rotometer measuring rate of sampling; M = glass wool filter

Detach the absorbers and wash the gas entry tube with 5 ml. of 10% ferric chloride solution in 1:1 hydrochloric acid. Dilute the contents of the second absorbers and washings to 15 ml. with water and read after 30 minutes at 540 mμ against a reagent blank. From this subtract one-third of the determined but uncorrected value of acetic anhydride as a correction to give the value for ketene.

Add 10 ml. of hydroxylamine reagent to the toluene from the first absorber. Shake for 5 minutes and let separate. Dilute an aliquot of the aqueous layer to 10 ml. with hydroxylamine reagent and add 5 ml. of the ferric chloride reagent and mix. Read against a reagent blank at 540 mμ after 30 minutes. Multiply the determined value for acetic anhydride by 1.33 as a correction to give the acetic anhydride.

SORBIC ACID, HEXADIENOIC ACID

Sorbic acid is steam-distilled from a magnesium sulfate-citric acid medium. Thereafter there are three methods of determination.[17] It may be read directly at 260 mμ.[18] It may be developed by reaction with hydroquinone in hot concentrated sulfuric acid. It may be developed with Wijs iodine solution. An alternative condition for distillation as applied to margarine replaces the magnesium sulfate with potassium bisulfate with a recovery of 96%.[19]

A green is developed with acetic anhydride and 2-methylthiobenzothiazole *p*-ethyltoluenesulfonate at 135°.[20] There is no interference by benzoic acid, salicylic acid, nipagin, formic acid, propionic acid, cinnamic acid, sulfuric acid, or parasorbic acid.

Procedure—*Food. Direct reading.* Steam-distil a sample containing 0.5-25 mg. of sorbic acid with 50 ml. of water, 50 grams of magnesium sulfate, and 1 gram of citric acid. Dilute to a known volume and read at 260 mμ.

Alternatively extract a sample with acidified aqueous methanol.[21] Read the filtrate at 252, 258, and 264 mμ. Then to correct for background $d_{258} = E_{258} - (E_{252} + E_{264})$. So $c = d_{258}/16.5\ l$ where l is the length of the absorption tube since $d_{258} = 16.5$ at 1 gram per liter and a tube length of 1 cm.

By hydroquinone. Alternatively, evaporate the steam distillate to 50 ml. and extract three times with 25-ml. portions of dichloromethane. Evaporate the combined extracts to dryness and treat the residue with 1 gram of hydroquinone in 2 ml. of concentrated sulfuric acid for 30 minutes at 130°. Cool, add 15 ml. of water, and extract with two 20-ml. portions of benzene. Shake the extract or an aliquot with 10 ml. of 16% sodium hydroxide solution and read the aqueous alkaline layer at 710 mμ.

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[17] H. J. Hardon and D. Visser, *Z. Lebensm.-Untersuch. u. -Forsch.* **108**, 397-402 (1958).

[18] D. Melnick and F. H. Luckmann, *Food Research* **19**, 20-7 (1954); F. H. Luckmann and D. Melnick, *Ibid.* **20**, 649-54 (1955); I. W. Diemair and K. Franzen, *Z. anal. Chem.* **166**, 246-53 (1959).

[19] H. Onrust, *Chem. Weekblad* **54**, 498-9 (1958).

[20] W. Dieman, *Naturwissenschaften* **44**, 180-1 (1957); I. W. Diemair and K. Franzen, *Z. anal. Chem.* **166**, 325-33 (1959).

[21] J. B. Roos and A. Versnel, *Chem. Weekblad* **55**, 521-5 (1959).

By Wijs reagent. As the third alternative, dilute the dichloromethane extract of the steam distillate to 100 ml. with the solvent. Dry with anhydrous magnesium sulfate. Treat an 8-ml. aliquot with 2 ml. of Wijs reagent and store for 24 hours in the dark. Read at 520 mμ.

By 2-methylthiobenzothioazole p-*ethyltoluenesulfonate.* To a sample containing 3-5 mg. of sorbic acid add 100 grams of magnesium sulfate heptahydrate, 100 ml. of water, and 4 ml. of 1:3 hydrochloric acid. From this distil 90-100 ml. Add 2-3 drops of concentrated hydrochloric acid and extract with five successive 20-ml. portions of ether. Combine the extracts and add 5 grams of phosphorus pentoxide with cooling. Evaporate *in vacuo* and take up the residue in 5 ml. of acetic anhydride. Combine 30 mg. of the dry reagent, 50 mg. of anhydrous sodium acetate, and 1 ml. of the sample in acetic anhydride. Heat at 135° for 15 minutes and cool. Add 10 ml. of methanol, cool, and shake. Read at 652 mμ.

FATTY ACIDS

Copper and cobalt soaps of fatty acids dissolved in chloroform give blue and red-blue colors which are the basis for a photometric analysis. The method is specific for acids in the range of C_7 to C_{18} and is independent of acidic or basic impurities.[22] The copper salt of saturated and unsaturated fatty acids of the $C_{10} - C_{22}$ series also dissolves in chloroform in the presence of triethanolamine to form a stable blue color which follows Beer's law.[23]

Lower fatty acids such as acetic, butyric, and caproic acid give no precipitate under the conditions described and higher fatty acids such as behenic acid are not sufficiently soluble for the estimation to be carried out at room temperature. Acids which this method can be applied to include caprylic, capric, lauric, myristic, palmitic, stearic, heptanoic, nonanoic, undecanoic, and tridecanoic. Unsaturated fatty acids such as oleic acid and resin acids such as abietic acid are also estimated by these methods. The maximum absorption of different acid salts is at the same wave length.

At low pH little or no cobalt soaps precipitate. The precipitation increases as the pH increases until the acid is neutralized. At higher pH values the metallic hydroxides also precipitate which interferes with the chloroform extractions. However, the pH of the reacting solution is not critical in the range of 8-10. Beer's law is followed over a concentration range of 0.2-0.04 M for the cobalt soaps and 0.04-0.008 M for the copper soaps. Below these concentrations the solutions are too pale for estimation and above these concentrations, the solutions are too concentrated and the metallic soaps often precipitate on standing.

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[22] Cora W. Ayers, *Anal. Chim. Acta* **15**, 77-83 (1956).

[23] Yoji Iwayama, *Yakugaku Zasshi* **79**, 552-4 (1959).

Since the cobalt soaps are also soluble in 100-120° petroleum ether, this solvent may be substituted for chloroform using three extractions.

Benzoic, oxalic, succinic, and malonic acid either give no precipitate or it is insoluble in chloroform. Abietic acid interferes with the estimation by copper soaps but not by cobalt soaps. In general the absorption corresponds to that of copper and cobalt ions. Small amounts of sodium or potassium chlorides as soluble in methanol do not interfere.

Higher fatty acids are also determined as their uranyl soaps after treatment with potassium ferrocyanide solution.[24] It was developed for determination of fecal fatty acids in extracts.

An indirect method for the fatty oils, such as coconut and palm oil, in citronella oil is based on the degradation of the oils to glycerol and the quantitative oxidation of glycerol to formaldehyde and formic acid with acid potassium periodate.[25] Thereafter the formaldehyde is determined with phenylhydrazine and a ferric salt. Phenylhydrazine also removes the excess of periodic acid. The time of reaction in each stage of the procedure is critical. Beer's law is followed. The color is stable for 15 minutes.

Fatty acids, oxidized by potassium dichromate, are estimated by determination of the reduced dichromate.[26] The reaction is carried out at 15 lb. per square inch. In blood samples the contribution of cholesterol must be subtracted from the readings.

Oleic acid in the range of 0.2-1.5 mg. of lipids is determined by this method. Fatty acids are also determined as hydroxamic acids and by rosaniline reagent.[27] In the analysis of alkyd resins for benzoic acid, page 380, the fatty acids are separated in carbon tetrachloride solution. The effects of oxidation or bleaching with decolorizing earth are shown by the ratios of absorption at 232 and 270 mμ.[28]

Procedure—*As copper soaps.* Dissolve a weighed sample in 0.56% potassium hydroxide in methanol. Adjust the pH to 9 with methanolic potassium hydroxide solution and add methanol to bring the concentration of the fatty acid within the range of 0.008-0.04 M. Add 10 ml. of 2% copper nitrate solution to a 5-ml. aliquot. After the blue precipitate forms add 10 ml. of chloroform and 5 ml. of water to ensure complete separation and shake vigorously. Separate the blue chloroform layer and filter if it is cloudy. Read at 675 mμ against chloroform.

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[24] R. Goiffon, B. Goiffon, and F. Kerleo, *Ann. biol. clin.* **10**, 422-4 (1952).

[25] A. I. Biggs, *Anal. Chem.* **26**, 602-3 (1954).

[26] A. C. Kibrick and S. Skupp, *Arch. Biochem. and Biophys.* **44**, 134-9 (1953).

[27] D. Mendelsohn, *S. African J. Med. Sci.* **23**, 75-82 (1958).

[28] J. P. Wolff, *Olii minerali, grassi e saponi, colori e vernici* **36**, 303-6 (1959).

Alternatively, shake a 0.0005-0.02 M solution of the fatty acid in chloroform with 5 ml. of a reagent prepared by mixing 9 ml. of triethanolamine, 1 ml. of 1:19 acetic acid, and 10 ml. of 5% cupric nitrate solution. Read the blue chloroform solution.

As cobalt soaps. Follow the procedure for copper soaps substituting a 2% cobalt nitrate solution in methanol for the copper nitrate and adding 10 ml. of water in place of the 5 ml. of water before chloroform extraction. Read at 525 mμ.

Emulsions. Adjust the pH to 9-10 by addition of 5.6% ethanolic potassium hydroxide to a 10-ml. sample. Dilute to 25 ml. with methanol. Develop 5 ml. of this for fatty acid with copper salt.

As uranyl soaps. To prepare the buffer, add 25 ml. of a solution containing 10% of sodium acetate, 5% of sodium nitrate, and 5% of acetic acid to 75 ml. of acetone. To a 20-ml. neutral aliquot of fatty acid in acetone, add 1 ml. of the buffer for pH 4 and 4 ml. of a saturated uranyl nitrate solution in 75% acetone. Filter or centrifuge and wash the precipitate with 75% acetone. Add an equal amount of buffer adjusted to pH 5.5 by addition of 4% sodium hydroxide solution. Add water to dissolve the precipitated uranyl phosphate and collect the residual uranyl soap after 2 hours by filtration or centrifuging. Wash with the buffer at pH 5.5. Dissolve the precipitate in 10 ml. of 1:49 hydrochloric acid at 30°, and extract with diethyl ether. Treat an aliquot of the yellow solution with 0.5 ml. of 5% potassium ferrocyanide solution and read the red solution with a green filter after 15 minutes.

By periodic acid. Citronella oil. Wash a 10-ml. sample in order with dilute acid, dilute alkali, and water. Saponify the washed samples with 20 ml. of 2.8% potassium hydroxide solution in methanol for 1 hour. This solvent permits the final evaporation at a low temperature. Dilute the residual solution with a saturated sodium chloride solution, add dilute sulfuric acid to make the solution slightly acidic, and warm to evaporate methanol. Extract the residue with ether, make the aqueous solution alkaline, and re-extract with ether. Neutralize the solution and extract with ether two more times. Warm the aqueous solution to evaporate ether, cool, and dilute to give a concentration of 0.001-0.006% of glycerol. Oxidize a 2-ml. aliquot containing 0.001-0.006% of glycerol to formaldehyde by shaking 10 minutes with 1.25% potassium periodate solution in 1:35 sulfuric acid. Add 0.5 ml. of fresh 1% phenylhydrazine hydrochloride solution to destroy excess periodic and iodic acids. Shake well and let stand for 10 minutes. Add 0.5 ml. of 1% ferric ammonium sulfate solution to the phenylhydrazone and shake for 5-10 seconds. Quickly add 25 ml. of 1:5 sulfuric acid, shake well, and read after 1 minute at 520 mμ.

By dichromate. To the dry sample of fatty acid add 2 ml. of 0.49% aqueous potassium dichromate. Add 4 ml. of concentrated sulfuric acid and heat for 20 minutes at 15 pounds pressure. Cool and carefully add 3.5 ml. of water. Mix and dilute to 10 ml. Read at 600 mμ and correct for a reagent blank.

LINOLEIC, LINOLENIC, AND ARACHIDONIC ACIDS

Polyunsaturated fatty acids containing the *cis*-methylene-interrupted structure, which includes linoleic, linolenic, and arachiodonic acids, are oxidized by atmospheric oxygen in the presence of the enzyme lipoxidase. The absorption of the conjugated diene hydroperoxide is read at 234 mμ.[29] As little as 0.005 mg. of linoleic acid can be measured by this technic.

Hydrogen-ion concentration and organic solvents affect the enzyme activity. Therefore, the alkali used in the saponification of samples must be neutralized before addition of the enzyme, and heat and a gentle stream of nitrogen must be applied to remove any organic solvents. Up to 3% of ethanol does not interfere, and up to 5% of ethanol can be tolerated. However, with this ethanol concentration, the incubation period of the sample with the enzyme must be at least doubled.

Since the absorption of the blanks with blood plasma is too high to zero the spectrophotometer at 234 mμ, a wave length of 245 mμ is chosen for plasma samples. When high concentrations of saturated esters or fatty acids are present, the sample should be heated. Elaidolinolenic, 10,12-linoleic, oleic, and octanoic acids do not interfere, nor do hydrogenated fats. Beer's law is followed. Since the three polyunsaturated acids give the same specific extinction coefficient, the method is a measure of the total of these acids. The fatty acids are highly reactive and all manipulations of the sample must be carried out in vessels which are flushed with nitrogen. The saponifications are particularly sensitive.

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[29] Joseph MacGee, *Anal. Chem.* **31**, 298-302 (1959).

Samples—To prepare a molar buffer, dissolve 61.9 grams of boric acid and 25 grams of potassium hydroxide in 800 ml. of water while heating at 100°. After solution is complete let the buffer cool to room temperature with stirring. Adjust the pH to 9 with 5.6% potassium hydroxide solution or 1:10 hydrochloric acid. Dilute to 1 liter with water. To prepare a diluted buffer which is 0.2 molar, dilute 200 ml. of the molar buffer to 1 liter with water.

Free fatty acids. Mix 200 ml. of the molar potassium borate buffer with the sample and dilute with water to 1 liter. Use a 3-ml. aliquot containing 0.005-0.025 mg. of free polyunsaturated fatty acids.

Fatty acid esters. Saponify a sample containing 0.5 mg. of polyunsaturated fatty acid by mixing it with 1 ml. of 2.8% ethanolic potassium hydroxide solution and allow it to stand for at least 4 hours in the dark. Add 20 ml. of molar buffer solution and 1 ml. of 1:24 hydrochloric acid. Dilute to 100 ml. and develop a 3-ml. aliquot.

Blood plasma. Saponify a 0.1-ml. sample following the technic for fatty acid esters. Add 5 ml. of molar buffer and develop.

Microorganisms. Saponify 100 mg. of dried mycelium at room temperature for 24 hours with 2 ml. of 8.4% potassium hydroxide solution. Add 20 ml. of molar buffer and 6 ml. of 1:24 hydrochloric acid. Dilute to 100 ml. with water. Filter and dilute an aliquot ten times with 0.2 molar potassium borate buffer.

Plant seeds. Wheat. Weigh and slice five wheat kernels into 5 transverse sections each. Saponify with 4 ml. of 8.4% potassium hydroxide solution at 100° for 2 hours. Let stand in the dark for 24 hours. Add 40 ml. of molar buffer and 5 ml. of 1:24 hydrochloric acid. Dilute to 200 ml. with water. Filter and develop a 10-ml. aliquot.

Procedure—To prepare the active solution, dissolve 10 mg. of lipoxidase in 10 ml. of ice-cold 0.2-molar potassium borate buffer. Mix a 2-ml. aliquot of the sample with 8 ml. of 0.2 molar ice-cold buffer. To prepare boiled lipoxidase to be used with blank solutions, heat a 5-ml. portion of the active lioxidase solution at 100° for 5 minutes.

To test the activity of each batch of enzyme, which varies from batch to batch, saponify a cottonseed-oil sample following the procedure for fatty acid esters. To a 3-ml. aliquot add 0.1 ml. of boiled lipoxidase solution and mix. Use this solution to zero the instrument at 234 mμ. To another 3-ml. aliquot add 0.1 ml. of active lipoxidase solution, mix, and note the time. Read the second solution against the first at 234 mμ at 1 minute intervals. The absorbance should increase to a maximum level in the second solution in less than 5 minutes. If not, either prepare a more concentrated enzyme solution or use a more active enzyme.

To determine the total polyunsaturated fatty acids add 0.1 ml. of boiled enzyme solution, or 0.33 ml. for plant-seed samples, to a 3-ml. aliquot of the sample which is 0.2 molar in potassium borate buffer and contains 0.005-0.025 mg. of free polyunsaturated fatty acids. Mix. Also mix another 3-ml. aliquot with 0.1 ml., or 0.33 ml. for plant-seed samples, of active enzyme solution. Let stand at room temperature for 30 minutes. Set the instrument at zero with the first solution and read the reacted sample against the blank. In general read at 234 mμ. Read blood plasma samples at 245 mμ. Calculate the polyunsaturated fatty acid content as follows:

$$\%\text{ polyunsaturated fatty acids} = \frac{A \times 3964}{W}$$

in which

A = the difference in absorbance between the blank and the sample

W = the total micrograms of sample in a 3-ml. sample solution.

In the case of blood plasma samples which are read at 245 mμ, use a factor of 5460 in the calculation in place of 3964.

LIPIDS

Lipids are oxidized by chromic acid and read at 620 mμ.[30] Trichloroacetic acid and perchloric acid interfere. Therefore the extraction is performed with fat solvents, an ethanol-ether mixture as the first solvent and carbon tetrachloride-ether as the second solvent. Chloroform interferes.

For determination of lipids the glycerol can be oxidized to glyoxal (Vol. III, p. 64) with bromine water and condensed with resorcinol to give an orange to violet color.[31] The methods are of course applicable to other fats and oils.

An alternative is to oxidize to formaldehyde with iodate and determine with chromotropic acid.[32]

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[30] John Paul, *Analyst* **83**, 37-42 (1958).

[31] W. Weigel, *Fette, Seifen, Anstrichmittel* **57**, 486-90 (1955); Cf. **58**, 1038-41 (1956).

[32] H. Ignatowska and A. Michajlik, *Polski Tygodnik Lekarski* **13**, 1037-8 (1958).

Samples—*Tissues.* Follow the procedure under nucleic acid, nonsugar portions, page 422.

Procedure—*By chromic acid.* Shake the lipid fraction with 2 ml. of 1% sodium hydrogen carbonate solution, if necessary adding 2 ml. of ether in order to facilitate the separation of the layers. Separate and wash the sodium bicarbonate layer twice with 2-ml. portions of ether. Add the washings to the remaining layer. Adjust this layer to a measured volume and evaporate aliquots to dryness. Determine lipid phosphorus in one aliquot and total lipids in another aliquot.

By iodate. Serum. Reflux a 1-ml. sample for 5 minutes with 15 ml. of 95% ethanol and 5 ml. of ether. Filter and repeat the extraction twice. Evaporate the combined filtrates to dryness and take up with 3 ml. of water. Add a drop of *p*-nitrophenol indicator solution and decolorize with 1:2 sulfuric acid. To this glycerine solution add 0.4 ml. of 1:2 sulfuric acid and 2.5 ml. of 2% sodium iodate solution. After 5 minutes destroy the excess iodate with 2.5 ml. of 17% sodium acid arsenite solution. Determine the formaldehyde by chromotropic acid, page 252.

Lipid phosphorus. Digest the residue containing 0.0002-0.002 mg. of phosphorus with 0.5 ml. of 2:5 sulfuric acid and 0.5 ml. of 2:8 perchloric acid. Evaporate on a sand bath at 160° and complete the digestion over an open flame. To the digested sample add 0.5 ml. of 2.5% ammonium molybdate solution and 0.5 ml. of aminonaphtholsulfonic acid reagent containing 0.1% of 1-amino-2-naphthol-4-sulfonic acid, 5.95% of sodium bisulfite, and 0.2% of anhydrous sodium sulfite. Dilute to 5 ml. with water and heat for 10 minutes at 100°. Cool and read the blue color at 820 mμ. The final concentration of acid in the reagents must not be less than N or high results will be obtained.

Total lipid. To the dried residue containing 0.05-0.5 mg. of total fat, add 3 ml. of 2.5% silver dichromate solution in concentrated sulfuric acid. Heat at 100° for 15 minutes, cool, and add 3 ml. of water. Read the green color at 620 mμ.

Resin Acids

Resin acids are determined as their cobalt soaps by a method designed primarily for fatty acids.

Procedure—When resins contain abietic acid reflux a 0.5-gram sample with 10 ml. of 5.6% potassium hydroxide solution for 4 hours. Neutralize to pH 9-10 by dropwise addition of concentrated hydrochloric acid. Filter the precipitated potassium chloride and wash it with methanol. Dilute the filtrate to 100 ml. and develop a 5-ml. aliquot as cobalt soaps as described for fatty acids, page 299.

Fats and Oils

In most common fats and oils, the unsaturated acids have nonconjugated double bond systems which do not absorb in the ultraviolet. The nonconjugated acids are converted to their conjugated isomers by isomerization in glycerol or glycol with alkali so they may be read in the ultraviolet[33] (Vol. III, pp. 316-25). Improvements introduced include addition of absorptivity constants for arachidonic acid, improvement of transparency of the reagent by protection with an atmosphere of nitrogen, introduction of corrections for absorption by extraneous compounds, and increased sensitivity and accuracy in the determination of small proportions of conjugated and nonconjugated acids. The acids used as standards have also been improved.

This procedure may be applied to animal fats, vegetable oils, and purified fatty acids and esters where the unsaturation is due to linolenic, linoleic, and arachidonic acids. The methods are not strictly quantitative when applied to oxidized fats, hydrogenated oils, certain drying oils, fish oils, and oils containing unusual acids or pigments which may interfere.

In general, there are five variations of the alkali reagent method.[34] With a 6.5% potassium hydroxide-glycol reagent, the transparency of the reagent is protected with an atmosphere of nitrogen.

A second method, using an 11% potassium hydroxide-glycerol reagent, may be used in the presence of pentanoic acid.[35] Heating time varies from 25 minutes to 45 minutes, although there is no increase in precision or accuracy with a longer isomerization period.[36] Either glycerol or glycol is a suitable solvent.

To produce maximum conjugated triene, tetraene, and pentaene acids from the respective nonconjugated acids, a stronger alkali concentration of 21% is required with an isomerization period of 15 minutes at 180°.[37] Pentanoic acid may also be calculated by simultaneous equations and the specific extinction coefficient of a fat or oil at a selected wave length is equal to the sum of the specific extinction coefficients of the components. However, components of fats or oils containing hexanoic acid may not be calculated by these equations. When isomerizing the acids of butter, 21% potassium hydroxide gives higher absorption values than 7-10% potassium hydroxide.[38]

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[33] American Oil Chemist's Society Tentative Method Cd 7-48; Cf. J. Moretti and Mme. R. I. Cheftel, *Bull. soc. chim. biol.* **37**, 699-707 (1955).

[34] S. F. Herb, *J. Am. Oil Chemists' Soc.* **32**, 153-8 (1955).

[35] F. I. Collins and V. E. Sedgwick, *Ibid.* **33**, 149-52 (1956).

[36] David Firestone, *J. Assoc. Offic. Agr. Chemists* **37**, 833-42 (1954).

A micro method using the 21% potassium hydroxide-glycol reagent has been developed and a semimicro method using 11% potassium hydroxide-glycol.[39] Greater accuracy with all reagents is attained by establishing absorptivity constants of the reference standards at slit widths which give the greatest possible absorptivity. A source of error appears when absorptivity constants are based on polyunsaturated acids isolated by bromination-debromination rather than by physical means.[40]

Other methods for fats are based on their turbidity in water. Fats in foods are dissolved in a mixture of trichloroacetic acid and acetone. The fats are precipitated as an emulsion by water and the turbidity compared with standards.[41] Oils in paraffin oil are measured by a variation of this turbidity procedure.[42] The fatty esters after saponification are also determined as their fatty acids.

Sample—*Blood serum.*[43] Slowly add a 3-ml. sample to 10-15 ml. of 3:1 warmed aldehyde-free alcohol and peroxide-free ether mixture. Heat the extract to boiling and filter. Repeat the extraction twice and add 0.2 ml. of 0.1% alcoholic hydroquinone solution to the combined filtrates to prevent oxidation. Add 0.1 ml. of 50% potassium hydroxide solution. Evaporate to dryness at 100° over a period of approximately 45 minutes during which saponification occurs. Dissolve the soaps in 5 ml. of water and precipitate the fatty acids with 11:89 hydrochloric acid. Add 8-10 ml. of petroleum ether while rotating the flask. Tightly stopper and let stand overnight to assure complete solution of the fatty acids. Remove the petroleum ether layer and extract the aqueous residue with three 3-4 ml. portions of petroleum ether. Add 0.2 ml. of a 0.1% alcoholic hydroquinone solution to these extracts to prevent oxidation and add the extracts to the original petroleum ether extract.

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[37] S. F. Herb and R. W. Riemenschneider, *J. Am. Oil Chemists' Soc.* **29**, 456-61 (1952); *Anal. Chem.* **25**, 953-5 (1953).

[38] S. Mattsson and P. Swartling, *Milk Dairy Research (Alnarp) Rept.* **55**, 8 pp. (1958).

[39] H. F. Wiese and A. E. Hansen, *J. Biol. Chem.* **202**, 417-23 (1953).

[40] R. T. O'Connor, M. F. Stansbury, H. G. Demaré, and S. M. Stark, Jr., *J. Am. Oil Chemists' Soc.* **29**, 461-6 (1952).

[41] V. P. Fedotov, *Voprosy Pitaniya* **15**, No. 4, 51-2 (1956).

[42] C. Holsky and I. Banciu, *Petrol si. Gaze* **6**, 534-7 (1955).

[43] H. F. Wiese and A. E. Hansen, *J. Biol. Chem.* **202**, 417-23 (1953).

Reduce the volume to approximately 3 ml. at room temperature using a fan. Resaponify the fatty acids at room temperature with 3 ml. of freshly prepared 0.56% potassium hydroxide solution in ethanol and 3 ml. of water by emulsifying thoroughly. After 1 hour, centrifuge for a few minutes to completely separate the petroleum ether layer containing the unsaponifiable material. Remove the petroleum ether layer and extract the residual layer three times with small volumes of petroleum ether. Evaporate the aqueous solution of soaps to near dryness at 80° over 2-2.5 hours. Add 5 ml. of water and precipitate the fatty acids with 11:89 hydrochloric acid. Add 10 ml. of petroleum ether, shake, stopper tightly, and let stand overnight. Centrifuge to separate the layers and transfer the petroleum ether with a pipet to a weighed flask. Extract three additional times with petroleum ether and add 0.1 ml. of 0.1% alcoholic hydroquinone solution. Evaporate the petroleum ether to dryness at room temperature with a fan. Dry the flask in a desiccator overnight and weigh, subtracting the weight of the 0.1 mg. of added hydroquinone from the weight of the sample. Transfer to an isomerization tube with petroleum ether and evaporate the petroleum ether in a stream of nitrogen under suction and concentrate the fatty acids by washing down with 2 ml. of petroleum ether. Determine with 11% potassium hydroxide-glycol, semimicro method.

Procedure—*Isomerization with 11% potassium hydroxide-glycerol.* Prepare an 11% potassium hydroxide-glycerol reagent containing 17.5 grams of 85% potassium hydroxide pellets per 100 ml. of glycerol. Heat a portion to 100° to lower its viscosity. Heat an 8.6-ml. portion until the temperature reaches 180°, add 0.1-0.125 gram of sample, and shake vigorously for 3-5 seconds. Reheat briefly and shake for 2 minutes additional to ensure complete saponification and heat until the temperature of the mixture reaches 175°. Time the isomerization period for 45 minutes cool to 60°, and add 93.5 ml. of absolute methanol. Cover, heat at 65-70° and let stand for 1 hour to reach room temperature. The volume should be approximately 100 ml. at this point. For linolenic acid, adjust the instrument to zero with 5 ml. of reagent and read at 268 mμ. For linoleic acid, read at 233 mμ. If 1-cm. cells are used, dilute 10:1 and 100:1 with methanol.

Calculate the linoleic and linolenic acid as follows:

$$\% \text{ linolenic acid} = \frac{K_{268}}{48.6} \times 100$$

where K_{268} = observed extinction coefficient at 268 mμ
48.6 = standard extinction coefficient at 268 mμ

$$\% \text{ linoleic acid} = \frac{K_{233}}{93.9} \times 100$$

where K_{233} = extinction coefficient of linoleic acid and where

$$K_{233} = KT_{233} - \frac{58.6}{48.6} K_{268}$$

where KT_{233} = total observed extinction coefficient
93.9 = extinction coefficient of 100% linoleic acid at 233 mμ
58.6 = extinction coefficient of 100% linolenic acid at 233 mμ.

Isomerization with 21% potassium hydroxide-glycol.[44] To prepare the potassium hydroxide-glycol reagent, heat ethylene glycol to 190° for 10 minutes and let cool to 150°. Add enough potassium hydroxide to give a solution of 21% potassium hydroxide, which means add approximately 28 grams of 85% potassium hydroxide pellets to 100 grams of glycol. This must be under nitrogen. Heat to 190° for 10 minutes and cool to room temperature. Check a weighed aliquot by titration and adjust to 21 ± 0.1% by adding ethylene glycol dried by heating at 190° for 10 minutes. Store under nitrogen at approximately 40°. To a 5.5 × 5/8 inch reaction tube, add 5 grams of reagent and cover with oxygen-free nitrogen. Heat at 180° for 15 minutes and add a 1-10 mg. sample in a weighing cup. If larger samples are available, dissolve a weighed amount with a weighed quantity of lauryl alcohol so that a 10-30 mg. aliquot contains 1-3 mg. of sample. Remove from the bath, shake vigorously for 5 seconds, and then replace in the bath. Repeat the shaking every 30 seconds until the mixture appears clear and homogeneous. Heat for a total of 15 minutes from the time the sample is added and cool in cold water.

Dilute the isomerized mixture to a definite volume with absolute methanol and read at 233 mμ for linoleic acid, 233 and 268 mμ for linolenic acid, 233, 268, and 315 mμ for arachidonic acid, 233, 268, 315, and 346 mμ for eicosapentanonic acid 233, 268, 315 and 346 mμ for docosapentanoic acid and 233, 268, 315, and 346 mμ for 50% C_{20}-50% C_{22} acids.

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[44] S. F. Herb and R. W. Riemenschneider, *Anal. Chem.* **25**, 953-5 (1953).

Calculate linoleic, linolenic, arachidonic, and pentanoic acids as follows:

1. Samples containing C_{20} pentanoic acid:

linoleic acid, % $= 1.092\ k_{233} - 0.573\ k_{268} - 0.259\ k_{315} + 0.023\ k_{346}$
linolenic acid, % $= 1.105\ k_{268} - 0.879\ k_{315} + 0.307\ k_{346}$
arachidonic acid, % $= 1.650\ k_{315} - 1.554\ k_{346}$
C_{20} pentanoic acid, % $= 1.143\ k_{346}$.

2. Samples containg C_{22} pentanoic acid:

linoleic acid, % $= 1.092\ k_{233} - 0.573\ k_{268} - 0.259\ k_{315} - 0.126\ k_{346}$
linolenic acid, % $= 1.105\ k_{268} - 0.879\ k_{315} - 0.016\ k_{346}$
arachidonic acid, % $= 1.650\ k_{315} - 1.863\ k_{346}$
C_{22} pentanoic acid, % $= 1.984\ k_{346}$.

3. Samples containing pentanoic acid of unknown chain length (calculated at 50% C_{20}-50% C_{22} pentanoic acids).

linoleic acid, % $= 1.092\ k_{233} - 0.573\ k_{268} - 0.259\ k_{315} - 0.033\ k_{346}$
linolenic acid, % $= 1.105\ k_{268} - 0.879\ k_{315} + 0.190\ k_{346}$
arachidonic acid, % $= 1.650\ k_{315} - 1.667\ k_{346}$
pentanoic acid, % $= 1.449\ k_{346}$.

Isomerization with 11% potassium hydroxide-glycol. Semimicro. As a reagent, heat 280 grams of ethylene glycol with 35 grams of 85% potassium hydroxide pellets in a stainless steel beaker. Using a stainless steel stirrer, stir until the potassium hydroxide is dissolved and the temperature reaches 178°. Stir until all water is driven off and the temperature reaches 180°. Adjust by titration to 11 ± 0.1% potassium hydroxide.

Heat the reagent at 180° in an oil bath for 10-12 minutes. Add 2 ml. of hot reagent to the residue of the sample, shake 10 seconds, and immediately immerse in an oil bath. Heat at 180° for 25 minutes to conjugate the fatty acids and chill immediately. Dilute to 25 ml. with absolute methanol and read at 375, 347.5, 300, 270, and 235 mμ. Using the $E^{1\%}_{1\text{ cm.}}$ values given in Table 19 calculate the % of fatty acids present.

As fatty-acid soaps. Reflux a 0.5-gram sample with 10 ml. of 5.6% potassium hydroxide solution for one hour. Neutralize the excess alkali

TABLE 19. STANDARDS[a] FOR LINOLEIC, LINOLENIC, AND ARACHIDONIC ACIDS

Unsaturated fatty acid	*No. of determinations*	235 mμ $E_{1\,\text{cm.}}^{1\%}$	270 mμ $E_{1\,\text{cm.}}^{1\%}$	300 mμ $E_{1\,\text{cm.}}^{1\%}$	347.5 mμ $E_{1\,\text{cm.}}^{1\%}$	375 mμ $E_{1\,\text{cm.}}^{1\%}$
Linoleic	12	779 ± 19	0.0	0.0	0.0	0.0
Linolenic	10	524 ± 40	518 ± 32	0.0	0.0	0.0
Arachidonic	12	484 ± 24	405 ± 22	350 ± 16	45 ± 4	0.0

[a] The results represent means ± the standard deviation.

by dropwise addition of concentrated hydrochloric acid until the pH is in the range of 9-10. Filter the precipitated potassium chloride and wash it with methanol. Dilute the filtrate to 100 ml. Treat an aliquot of 5 ml. as described for fatty acids as copper or cobalt soaps but calculate as the ester, page 298.

LICANIC ACID, 4-OXO-OCTADECA-9,11,13-TRIENOIC ACID

Since the ultraviolet absorption spectra of licanic acid and eleostearic acid, octadeca-9,11,13-trienoic acid, are almost identical a measurement of absorption at 270.5 mμ gives only a measure of the total conjugated trienoic unsaturation. To determine licanic acid in the presence of eleostearic acid, the ketonic groups are measured with 2,4-dinitrophenylhydrazine reagent.[45]

Different batches of ethanol give different values because of the presence of variable amounts of water during the condensation reaction. To overcome this disadvantage, a control test must be simultaneously run on the pure ketone being determined or on some ketonic compound of similar nature which has been calibrated against the ketone under analysis.

Procedure—To prepare ketone-free ethanol, distil absolute ethanol from zinc dust and potassium hydroxide. Reflux 1.5 liters of the distillate with 10 grams of 2,4-dinitrophenylhydrazine and 10 ml. of concentrated sulfuric acid for 2 hours and distil off the ketone-free ethanol which appears yellow owing to the volatility of the 2,4-dinitrophenylhydrazine.

To prepare the 2,4-dinitrophenylhydrazine reagent, add 1 ml. of constant-boiling hydrochloric acid to 0.06 gram of 2,4-dinitrophenylhydrazine. Dissolve in 80 ml. of ketone-free ethanol by warming. Cool and dilute to 100 ml. with ketone-free ethanol.

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[45] A. Mendelowitz and J. P. Riley, *Analyst* **78**, 704-9 (1953).

Dissolve a 0.1-gram sample in 100 ml. of ketone-free ethanol and dilute to give a solution approximately 0.001% with respect to the ketonic group. Add 1 ml. of dinitrophenylhydrazine reagent to a 1-ml. aliquot, stopper loosely, and heat at 60° for 50 minutes. Tighten the stopper after 1 minute. Cool in water for 2 minutes, add 5 ml. of 10% potassium hydroxide solution in 80% ethanol, and dilute to 10 ml. with water. Read at the secondary absorption maximum at 535 mμ for 12-oxostearic acid and licanic acids against water. Correct for a reagent blank and a control of pure ketone compound or related compound.

KARATHANE, DINITROCAPRYLPHENYLCROTONATE

Upon hydrolysis dinitrocaprylphenylcrotonate yields crotonic acid and dinitrocaprylphenol, the latter of which is determined. Dinitrocaprylphenylcrotonate is separated with any free dinitrophenol from the sample by steam distillation, the distillate extracted with chloroform, and taken up in pyridine-water in which the hydrolysis takes place. The color is developed after 1 hour and read at 442 mμ. The method is sensitive to as little as 0.01 mg. in a 200-gram sample.[46]

Hexane-acetonitrile is used to remove fats in animal samples. If more than 0.5 gram of fat comes through the steam distillation the dinitrocaprylphenylcrotonate remains dissolved in the fat giving poor recovery. Excessive heating and too strong a nitrogen stream leads to low results. Parathion, carbamates, and chlorinated insecticides do not interfere.

Apparatus—Assemble the apparatus as shown in Figures 14 and 15. This apparatus contains, in addition to parts described in the diagram: two variable transformers, capable of controlling 0-120 volts of alternating current, one to control the steam generator and one to control the steam distillation flask; an electronic relay, 115 volts and 60 cycles, or any type which can handle an inductive load, and a fixed point thermoregulator, 130° ± 1° with a ₸ 10/30 joint and a 2-inch immersion stem.

Samples—*Vegetable matter. Surface extraction.* Add 1 ml. of benzene for each 2 grams of sample to a sample of approximately 1000 grams. If the sample is exceptionally dry, increase the benzene ratio. Mix for 45 minutes in a tumbler and decant the extract. Filter if necessary through

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[46] I. Rosenthal, C. F. Gordon, E. L. Stanley, and M. H. Perlman, *J. Agr. Food Chem.* 5, 914-8 (1957).

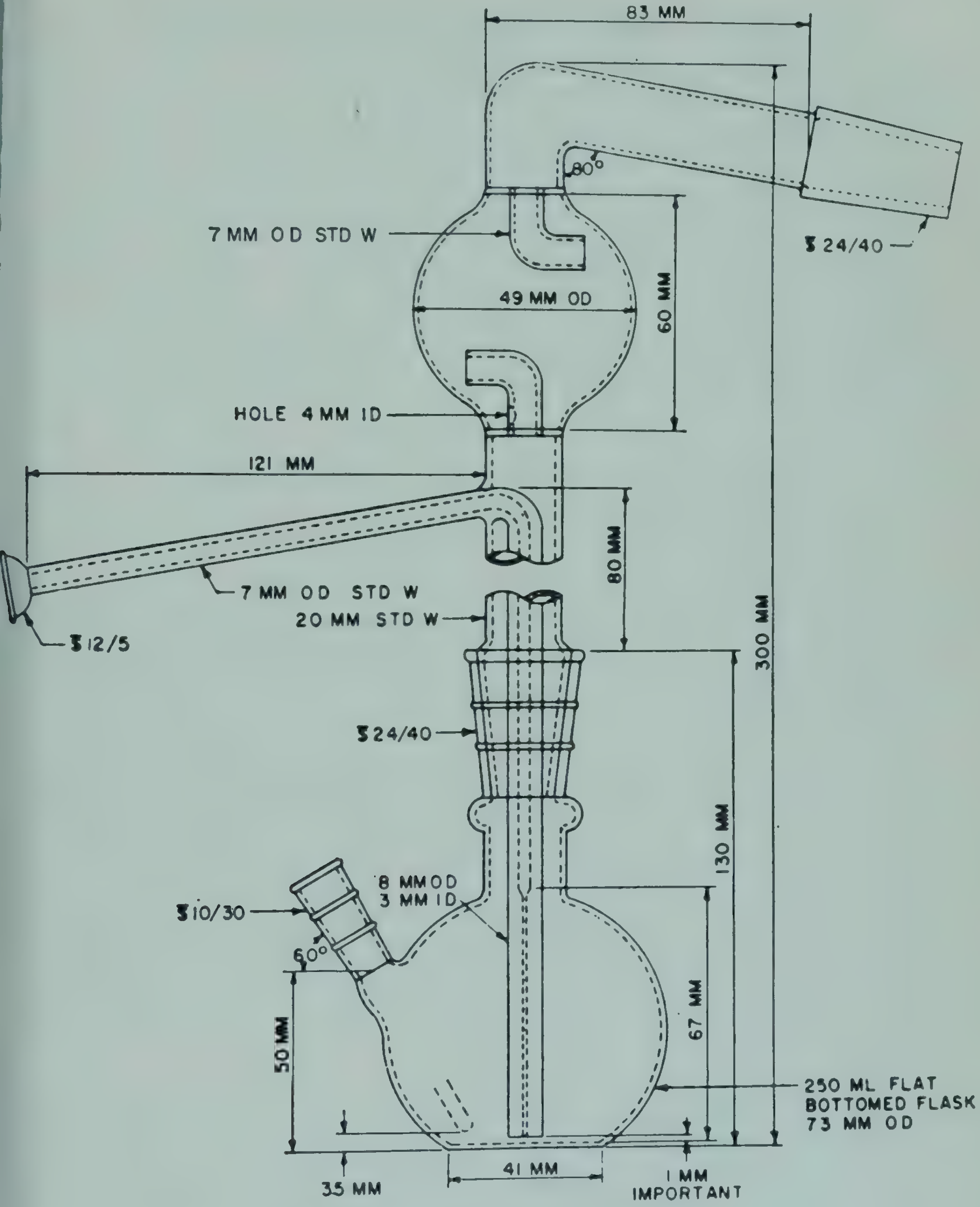
83 MM
80°
7 MM OD STD W
₮ 24/40
49 MM OD
60 MM
HOLE 4 MM ID
121 MM
80 MM
7 MM OD STD W
20 MM STD W
₮ 12/5
300 MM
₮ 24/40
130 MM
8 MM OD
3 MM ID
₮ 10/30
60°
50 MM
67 MM
250 ML FLAT
BOTTOMED FLASK
73 MM OD
41 MM
35 MM
1 MM
IMPORTANT

Fig. 14

Distillation flask and head for samples containing dinitrocaprylphenol as used in Figure 15

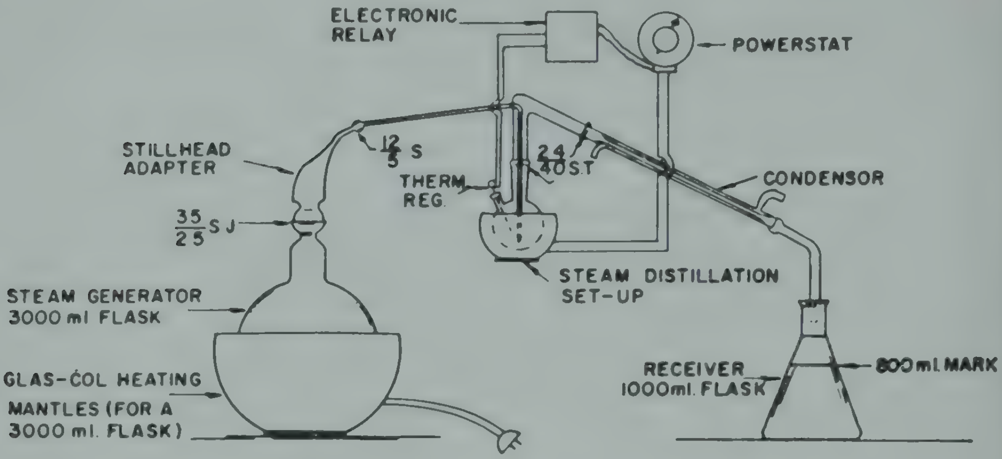
ELECTRONIC RELAY
POWERSTAT
STILLHEAD ADAPTER
12/5 S
24/40 S.T
THERM REG.
CONDENSOR
35/25 S J
STEAM DISTILLATION SET-UP
STEAM GENERATOR 3000 ml FLASK
RECEIVER 1000ml. FLASK
800 ml. MARK
GLAS-ĆOL HEATING MANTLES (FOR A 3000 ml. FLASK)

FIG. 15.

Steam-distillation apparatus for dinitrocaprylphenol

glass wool or cheesecloth. Seal with an aluminum foil-lined cap and store at 10° until analyzed.

For soft, fragile fruits such as tomatoes, cherries, and strawberries, agitate by rolling for 10 minutes. Let stand for 15 minutes, roll for 10 minutes, and let stand for 10 minutes. Separate the extract and store at 10° until analyzed.

Animal tissues. Macerate a thawed sample with Ottawa sand, using 5 grams of sand per gram of sample. Transfer the mixture to an extraction thimble and place in a Soxhlet extractor. Wash the mortar and pestle with two 10-ml. portions of diethyl ether and slowly add to the thimble. Add 55 ml. of diethyl ether to the flask and extract the sample for 4 hours. Evaporate the ether extract just to dryness and dissolve the residue in 30 ml. of *n*-hexane. Extract four times with 30-ml. portions of acetonitrile. Extract the combined acetonitrile layers with 30 ml. of *n*-hexane and discard the *n*-hexane extracts.

Vegetable matter. Feed a 500-gram sample into a blender at such a rate that the fluidity is maintained. During grinding of the pulp, the temperature should not exceed 30°. In the case of apples and pears, add 250 ml. of ice water before the initial blending to cool the reagents. If the mixtures go above 30° during grinding, jacket the blender with a plastic film containing dry ice.

Grind for 5 minutes and add 10 ml. of 1:1 sulfuric acid. Mix for 2 minutes and add 400 ml. of benzene. Blend for 5 minutes, add 100 grams of anhydrous sodium sulfate, and mix for two minutes. Centrifuge for 20 minutes and collect the supernatant benzene layer. Redisperse the solids and recentrifuge. Add any additional supernatant liquid to the original extract as the sample.

Procedure—Evaporate the solvent from an aliquot of the sample containing up to 0.13 mg. of dinitrocaprylphenylcrotonate in the steam distillation apparatus shown in Figure 14 to a volume of 1-2 ml. at 100° under a gentle stream of nitrogen. While the flask is still hot, drive off the remaining solvent with the nitrogen stream. Bring the steam generator to a boil and set the Powerstat so that 800 ml. of distillate are collected in 1 hour, or about 110 volts. Add 15 ml. of 1:1 sulfuric acid to the distillation flask and connect the apparatus so that it operates automatically at 130° ± 1°. Collect 775 ml. of distillate, allow the condenser to steam down to flush out any waxes or fats that may have solidified in the condenser, and collect the final 25 ml. Remove the receiver before the heat is shut off to prevent suck-back.

Separate the distillate for extraction. Rinse the receiver with 10 ml. of 2B ethanol or 95% ethanol followed by two 50-ml. portions of chloroform and add to the distillate. Shake for 2 minutes, remove the chloroform, and evaporate it to dryness. Add 3 ml. of a 1:1 pyridine-water mixture to the residue to hydrolyze the dinitrocaprylphenylcrotonate, rinsing down the sides of the flask. Stopper, swirl, and read the yellow solution after 1 hour at 442 mμ against a reagent blank. Correct for a control sample blank prepared from untreated samples.

CHAPTER 14

SUBSTITUTED MONOBASIC ALIPHATIC ACIDS AND THEIR ESTERS

o-Hydroxyphenylacetic acid is determined by nitrosation of the phenol, page 99. The determination of 2,4-*D* acid by chromotropic acid, page 325, has been applied successfully to 4-chloro-*o*-toloxyacetic acid, MCP acid. For determination of 3-(*o*-tolyloxy) lacetic acid in plasma and urine see 3-*o*-tolyloxy-1,2-propanediol, Mephenesin, page 47. For determination of ascorbone with ascorbic and diketogulonic acid see page 346. Because determined by its phenolic properties, *o*-hydroxyphenylacetic acid is given on page 98.

The maximum for direct reading of levulinic acid, acetopropionic acid, is at 270 mμ.[1] Important interferences are by 5-hydroxymethyl-2-furoic acid, bis(5-formyl-2-fufuryl) ether, furfural, and 5-hydroxymethyl-2-furfural. A lesser interference is by 2,5-bishydroxymethylfuran.[2]

For determination of diketogulonic acid in the presence of ascorbic acid and ascorbone see page 346. For the direct reading of desoxycholic acid and cholic acid in the ultraviolet, see cholic acid, page 350. They are also determined as hydroxamic acids by ferric perchlorate, page 350.

5-Hydroxymethyl-2-furoic acid is read directly at 249.5 mμ. Interferences are bis(5-formyl-2-furfuraldehyde) ether, furfural, 5-hydroxy-2-furfural, and 2,5-bishydroxymethylfuran. For direct reading of styrenated fatty acids see page 15.

Carboxylic Acids

The substitution on the carboxylic acid molecule influences the rate of its reaction with hydroxylamine hydrochloride to form hydroxamic acids which are read after reaction with ferric iron.[3] Bulky groups present steric hindrance while electron-donating groups also inhibit the hydroxamic acid formation. α-Chloro, α-amino, and α-hydroxy substituents do not inhibit hydroxamic acid formation but they exert a hypochromic effect on the color reaction between ferric iron and the hydroxamic acids. The speed of hydroxylamine reaction of a series of ortho-substituted and para-substituted methyl benzoate esters varies approximately inversely with the electron releasing-strength of these substituents:

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[1] G. Mackinney and O. Temmer, *J. Am. Chem. Soc.* 70, 3586-90 (1948).

[2] J. H. Turner, P. A. Rebers, P. L. Barrick, and R. H. Cotton, *Anal. Chem.* 26, 898-901 (1954).

[3] V. Goldenberg and P. E. Spoerri, *Ibid.* 30, 1327-30 (1958).

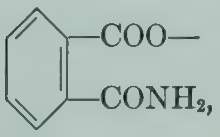
para-substituted $O^- \ll NH_2 < CH_3$

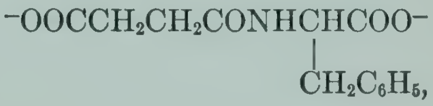
ortho-substituted $O^- < COO^- \ll NH_2, \quad CH_3 < OCH_3.$

Monocarboxylic acid amides which have no electron-withdrawing substituents react very slowly with hydroxylamine reagent. Replacement of one hydrogen of formamide by a methyl, higher alkyl, or phenyl group results in retardation of the hydroxylamine reaction as follows:

$HCONH_2 = FCH_2CONH_2 > CH_3CONH_2 =$

$^-OOCCH_2CH_2CONH_2 > CH_3CONHC_6H_5 > CH_3CONHCH_3,$

$C_6H_5CONH_2 >$ 



$^-OOCCH_2CH_2CONH\underset{CH_2C_6H_5,}{\underset{|}{C}}HCOO^-$

$HCON(CH_2CH_3)_2$

Procedure—*As hydroxamic acids.* To prepare the hydroxylamine reagent, mix equal volumes of refrigerated 14% sodium hydroxide solution and refrigerated 14% hydroxylamine hydrochloride solution and rapidly bring to room temperature by immersing in a water bath. Immediately add 2 volumes of reagent to 1 volume of ester or amide solution which is 0.0025*M* with respect to the ester or amide prior to adding the reagent. Maintain at 25° or at room temperaure for the time required, see Table 20.

To a 3-ml. aliquot add 1 ml. of 1:2 hydrochloric acid and exactly 1 minute later add 1 ml. of 20% ferric chloride solution in 1:110 hydrochloric acid. Read at 540 mμ.

Glycolic Acid

In the determination of glycolic acid with 2,7-dihydroxy-naphthalene in the presence of sulfuric acid (Vol. III, p. 328) glycol must be removed, since any present will be oxidized to glycolic acid.[4] At the maximum absorbance at 535 mμ the calibration curve shows a slight deviation from

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[4] H. Green, *Analyst* 82, 107-10 (1957).

Table 20. Reaction Time for Maximum Hydroxamic Acid Formation

Monocarboxylic acid ester	*Minutes*
Ethyl acetate	2
Methyl propionate	2
Methyl butyrate	2
Monomethyl succinate	2
Ethylbenzoate	2
Methyl *o*-methoxybenzoate	2
n-Butyl *o*-methoxybenzoate	15
Methyl *p*-hydroxybenzoate	60
Methyl salicylate	40
n-Butyl salicylate	70
Monomethyl phthalate	28
Mono *n*-butyl phthalate	120
Methyl *o*-toluate	10
Methyl *m*-toluate	2
Methyl *p*-toluate	2
Methyl anthranilate	9
Methyl *p*-aminobenzoate	7
Acetylsalicylic acid	2
Alkyl and phenylalkyl carboxylic acid esters	
l-Phenylalanyl ethyl ester	2
Glycine ethyl ester	2
Acetyl-*dl*-phenylalanyl ethyl ester	2
l-Tyrosine ethyl ester	2
Ethyl lactate	2
Ethyl *l*-β-phenylacetate	2
Ethyl *dl*-β-hydroxy-*n*-butyrate	2
Methyl methoxyacetate	2
Ethyl-β-ethoxypropionate	2
Ethyl chloroacetate	2
Monocarboxylic amides	*Hours*
Formamide	1
Fluoroacetamide	1
N-methylacetamide	24
Acetamide (0, 005-0.01 *M*)	6 to 8
Acetamide (0.0025 *M*)	6
Acetanilide	9
N-maleyl-*dl*-phenylalanine	8 with no maximum hydroxamic acid formation
Phthalamidic acid	19 with no maximum formation
N-(*o*-carboxybenzoyl)-*dl*-phenylalamine	18 with no maximum acid formation
Succinamic acid	6.5

Beer's law. Both free and combined glycolic acid are determined by this procedure. The reagent should stand 24 hours before use, in which time it turns blue.

Glycolate is washed out of carboxymethylcellulose with 80% ethanol for determination by chromotropic acid.[5] Sodium chloride, acetic acid, oxalic acid, monochloracetic acid, dichloroacetic acid, and caustic extract of cellulose do not interfere. Beer's law is followed for 0.01-0.1 mg. per ml. of glycolic acid.

Sample—*Used antifreeze solutions of the inhibited ethylene glycol type.* Mix a 10-ml. sample with 40 ml. of water and 5-10 grams of zinc oxide to remove insoluble matter. Centrifuge for 5 minutes. Stir 25 ml. of the supernatant liquid with 1-2 grams of solid cation-exchange resin, Zeo-Karb 225, to remove zinc, iron, and soluble cations. Let stand, filter, and wash. Add 1 gram of an anion-exchange resin, dry De Acidite FF, to the filtrate, which contains ethylene glycol and glycolic acid, to absorb the glycolic acid. Mix well, let stand, and filter on a sintered crucible. Wash with water and dry the crucible with filter paper. Remove glycolic acid from the resin with two 5-ml. portions of 4:1 sulfuric acid. Add 20 ml. of concentrated sulfuric acid and mix. Develop by 2,7-dihydroxynaphthalene.

Crude sodium carboxymethylcellulose. Wash a 3-gram sample with 30-50 ml. portions of 80% ethanol at 50-70° until chloride-free. This requires approximately 200-600 ml. of 80% ethanol depending on the salt content of the carboxymethylcellulose. Dilute so that a 1-ml. aliquot contains 0.04-0.08 mg. of sodium glycolate and develop by chromotropic acid.

Procedure—*By 2,7-dihydroxynaphthalene.* To a 5-ml. aliquot in nearly concentrated sulfuric acid, add 10 ml. of 0.01% 2,7-dihydroxynaphthalene solution in concentrated sulfuric acid. Heat for 10 minutes, cool, and dilute to 25 ml. with 1:1 sulfuric acid. Read at 525 mμ against a blank containing 10 ml. of 4:1 sulfuric acid and 20 ml. of concentrated sulfuric acid treated similarly.

By chromotropic acid. To a 1-ml. aliquot containing 0.04-0.08 mg. of sodium glycolate in 80% ethanol, add 0.5 ml. of 5% chromotropic acid solution and 20 ml. of concentrated sulfuric acid. At the same time prepare a reagent blank using 1 ml. of water in place of the sample. Stopper and mix. Loosen the stoppers and heat at 100° for 30 minutes. Cool to room temperature, dilute to 25 ml. with concentrated sulfuric acid, and mix. Read against the reagent blank at 570 mμ.

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[5] Marie Easterwood, *Anal. Chem.* **29,** 981-2 (1957).

CYANOACETIC ACID HYDRAZIDE

A reagent for cyanoacetic acid hydrazide is *p*-dimethylaminobenzaldehyde in sulfuric acid and ethanol.[6]

Procedure—*Blood.* Deproteinize an oxalated blood sample with 20% trichloroacetic acid solution. Filter and add a 10-ml. portion to 1 ml. of a reagent prepared from 1 gram of *p*-dimethylaminobenzaldehyde in 4 ml. of concentrated sulfuric acid and 15 ml. of 95% ethanol. Heat for 45 minutes at 100°, and read.

THIOGLYCOLIC ACID

Thioglycolic acid reacts with sodium nitroprusside in alkaline medium to yield a red-violet color which is a function of the temperature, pH, and time.[7]

Nitrous acid is another reagent for thioglycolic acid.[8] In the range of 0.5-2 mg. per 10 ml., thioglycolic acid is read at 330 mμ while larger amounts in the range of 10-40 mg. per 10 ml. are read at 545 mμ. In both spectral regions absorption is closely proportional to the thioglycolic acid concentration but the optical density at 330 mμ is 35-50 times that at 545 mμ for a given amount of acid.

Thioglycolic acid is developed in buffered solution with 2,6-dichlorophenolindophenol and extracted into xylene. Reducing agents interfere.[9]

Procedure—*By sodium nitroprusside.* Dilute a 10-ml. sample to 1 liter and mix a 10-ml aliquot with 70 ml. of water at 15°. Add 10 ml. of 0.4% sodium hydroxide solution and 10 ml. of 1% sodium nitroprusside solution. Read within 2 minutes.

By nitrous acid. Treat a 10-ml. aqueous aliquot containing 0.5-2 mg. of thioglycolic acid with 10 ml. of 1% sodium nitrite solution and 5 ml. of 1:100 hydrochloric acid. Dilute to 25 ml. and read within 10 minutes. For concentrations of 0.5-2 mg. per 10 ml. of sample read at 330 mμ against a reagent blank. For concentrations of the order of 10-40 mg. per 10 ml. of sample read at 545 mμ.

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[6] K. Maresch and E. Philippi, *Wien med. Wochschr.* 106, 505-6 (1956).

[7] F. Provvedi and S. Camozzo, *Chim. e ind.* (*Milan*) 34, 517-9 (1952).

[8] F. N. Woodward, *Analyst* 74, 179-82 (1948); G. T. Walker and F. M. Freeman, *Mfg. Chemist* 26, 11-3 (1955).

[9] S. Terada and H. Nomiyama, *Eisei Kagaku* 4, 31-3 (1957).

By 2,6-dichlorophenolindophenol. Mix a 5-ml. sample containing 0.01-0.02 mg. of thioglycolic acid with 5 ml. of buffer prepared from 30 grams of sodium acetate, 70 ml. of water, and 100 ml. of glacial acetic acid. Add 5 ml. of 2,6-dichlorophenolindophenol solution prepared from 160 mg. of 2,6-dichlorophenolindophenol in 350 ml. of water. Add 10 ml. of xylene and shake well for 20 seconds. Centrifuge and read the xylene layer at 500 mμ.

3-Methoxy-4-hydroxymandelic Acid, Vanilmandelic Acid

With dilute sulfuric acid and activated alumina as a catalyst, 3-methoxy-4-hydroxymandelic acid is converted to vanillin, which is developed with indole in the presence of orthophosphoric acid.[10] Since urine samples contain an inhibitor which prevents the breakdown of the acid to vanillin, these samples are passed through a cation-exchange resin, eluted with ammonium acetate at pH 4.8, and the acid extracted from the eluate with ethyl acetate.

Procedure—*Urine.* Prepare an ammonium acetate buffer at pH 5.9 by dissolving 115.7 grams of ammonium acetate in 400 ml. of water. Adjust the pH to 5.9 with glacial acetic acid and dilute to 500 ml. with water. Prepare an ammonium acetate buffer at pH 4.8 by dissolving 38.5 grams of ammonium acetate in 400 ml. of water. Adjust the pH to 4.8 with glacial acetic acid and dilute to 500 ml. with water.

To convert the cation-exchange resin from the chloride to the acetate, slurry 100 grams of Dowex 1 X2 resin with 43 ml. of ammonium acetate buffer at pH 5.9. Pour into a 2.5 × 50 cm. glass column and wash with buffer at pH 5.9 until the eluate is chloride-free, using about 300 ml. of buffer. Wash with 500 ml. of ion-exchange water and drain. Slurry 1.5 grams of the resin with 1 ml. of ion-exchange water and pack into a 25 × 0.5 column. Run off the excess water and add a 1-ml. sample. Let stand for 2-3 minutes and elute the 3-methoxy-4-hydroxymandelic acid with 15 ml. of ammonium acetate buffer at pH 4.8 over a period of 40-50 minutes. Acidify the eluate with 2 ml. of 1:1 sulfuric acid, add 8 grams of sodium chloride, and extract the acid by shaking for 5 minutes with 15 ml. of ethyl acetate. Centrifuge and separate the upper ethyl acetate phase. Repeat the extraction with 15 ml. of ethyl acetate. Concentrate the first ethyl acetate extract, add to the second extract, and distil off the solvent under reduced pressure at 40°. All acetic acid should be removed and the residue should not smell of acetic acid. Add 2.5 ml. of 1:19 sulfuric acid and 0.2 gram of activated alumina. Stopper, separating the stopper from the neck of the tube with a thread, and autoclave at 15 psi for 1 hour to convert the acid to vanillin. Mix thoroughly and centrifuge. As a reagent, mix 2.4 ml. of 85% orthophosphoric acid and 0.1 ml. of 0.5% indole solution in ethanol. Add 1.5 ml. of the supernatant liquid, mix gently, and read the pink color at 495 mμ within 5 minutes.

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[10] M. Sandler and C. R. J. Ruthven, *Lancet* **1959-II,** 114-5.

MONOBROMOACETIC ACID

Monobromoacetic acid forms a red thioindigo by reaction with thiosalicylic acid in alkaline medium.[11] The compound is soluble in chloroform.

Procedure—As a reagent dissolve 1.5 grams of thiosalicylic acid in 8 ml. of 40% sodium hydroxide solution and dilute to 100 ml. To a 2-3 ml. sample add 2 drops of reagent and evaporate to dryness at 100-110°. Heat for 15 minutes at 130-140°. Dissolve in 4-5 ml. of water and add 2% potassium ferrocyanide solution dropwise until a maximum color is reached. Extract with chloroform and read.

TRICHLOROACETIC ACID

A crimson color is formed when trichloroacetic acid is added to a boiling mixture of pyridine and strong aqueous alkali (Vol. III, p. 336).[12] Since on heating the sodium salt of trichloroacetic acid decomposes readily to chloroform it follows that this is a determination of chloroform.[13] It follows as expected that chloroform yields a color of equal absorbance. Chloral hydrate gives a color with an absorbance 85% of that of trichloroacetic acid, and trichloroethylene, a crimson color which gradually fades to yellow with an absorption maximum at 400 mμ.[14] The interference of chloroform and trichloroethylene is eliminated by preliminary aeration of the sample. Trichloroethanol and urochloralic acid do not interfere.

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[11] M. Peronnet and S. Rocques, *Ann. fals. et fraudes* **46,** 21-3 (1953).

[12] T. W. Tibbits and L. G. Holm, *J. Agr. Food Chem.* **1,** 724-6 (1953).

[13] G. L. Mills, *Anal. Chim. Acta* **7,** 70-5 (1952).

[14] T. A. Seto and M. O. Schultze, *Anal. Chem.* **28,** 1625-9 (1956).

The method is applicable to determination of trichloroacetic acid in monochloroacetic acid.[15] Dichloroacetic acid is converted to glyoxal and does not interfere.

In the analysis of urine samples, the optimum alkali concentration for urine of different species must be determined in each case. In the analysis of plant materials fat-soluble pigments such as chlorophyll and carotenoids are precipitated at pH 3.5 by acetic acid. They are filtered with other insoluble cellular components on asbestos. For determination of total trichloro compounds including trichloroacetic acid in urine see page 483.

Procedure—*Urine.* To a 1-ml. sample, diluted if necessary, add 1 ml. of toluene, 5 ml. of 18% potassium hydroxide solution, and 10 ml. of pyridine. Mix gently, attach an air condenser, and heat at 64-65° without agitation for 40 minutes. Cool in ice for 3-5 minutes. Clarify a 5-ml. aliquot of the pyridine layer by addition of 1 ml. of water and read at 530 mμ within 30 minutes.

Plant material. Cut a weighed sample into approximately 2-mm. squares and homogenize with 2 ml. of 1:190 acetic acid for each 500 mg. of sample until the sample is of a creamy consistency. Filter through asbestos with moderate suction to eliminate pigments such as chlorophylls and carotenoids, and insoluble cellular components. Adjust the pH of a 1-ml. aliquot to 8-10 with approximately 0.1 ml. of 4% sodium hydroxide solution. The appearance of a flocculent protein precipitate is an indication of alkalinity. Refilter through asbestos.

Add a 0.2 ml. sample containing 0.006-0.024 mg. of trichloroacetic acid and 0.025 ml. of a saturated sodium hydroxide solution to 3 ml. of pyridine. Stopper with a cork and shake vigorously 10 times. Remove the cork and heat for 5 minutes at 100° to develop the color. Cool at 25° for 3 minutes. Read the pyridine layer within 15 minutes at 510 mμ against a reagent blank containing a filtrate from a sample which has not been treated with trichloroacetic acid.

Phenylacetic Acid

The nitration of phenylacetic acid followed by reduction with hydroxylamine in ammonical solution produces a stable violet color suitable for photometric measurement. Since nitration at 96-98° produces at least five different nitro compounds, the nitration is carried out at 40-44° to eliminate the formation of interfering derivatives.[16] Phenylacetic acid, a precursor in the biosynthesis of benzylpenicillin, is extracted from benzylpencillin or its degradation products by acidified solutions with toluene or chloroform. The presence of sodium sulfate shifts the distribution of equilibrium in favor of toluene probably as a salting-out effect. Phenylacetic acid can be separated from phenylacetamides by extraction of the phenylacetamides with chloroform from an alkaline solution.

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[15] N. Bellen and M. Jurowska-Wernerowa, *Przemysl Chem.* 11, 526-7 (1955).

Amounts as small as 0.05 mg. per ml. are measured by this procedure. It is possible to determine 0.01 mg. per ml. by a modification of the general procedure in which *o*-hydroxyphenylacetic acid, an interference in the modified procedure, is oxidized by potassium ferricyanide, rendering it insoluble in toluene. This modified procedure produces a ±3% error, and is not applicable in the presence of phenylacetamides. Beer's law is followed. Considerable error due to excessive heating and splattering is avoided by using a small amount of nitrating reagent and a diluted ammonium hydroxide reagent. The color formation from *dl*-mandelic acid is reduced by the addition of water to the nitrating reagent. Phenylalanine, tryptophan, tyrosine, and histidine do not interfere.

Sample—*Benzylpenicillin, benzylpenicillic acid, and benzylpenicilloic acid solutions.* Acidify a 1-ml. aliquot with 0.1 ml. of 1:3 sulfuric acid and add 0.25-0.3 gram of anhydrous sodium sulfate. Shake for 30 seconds with 5 ml. of toluene. Do not use a rubber stopper. Stopper loosely and centrifuge for 5 minutes to separate the solvent layers. Take an aliquot containing up to 0.5 mg. of phenylacetic acid.

If phenylacetamides are present, make the sample alkaline with 0.2 ml. of 4% sodium hydroxide solution and add 0.6 gram of anhydrous sodium sulfate. Shake for 30 seconds with 10 ml. of chloroform. Stopper loosely and centrifuge for 5 minutes. Remove the chloroform layer and repeat the extraction twice. Discard the chloroform layers containing phenylacetamides.

Procedure—*About 0.05 mg. per ml.* Alkalize an aliquot containing up to 0.5 mg. of phenylacetic acid with 0.1 ml. of 0.4% sodium hydroxide solution. Dry at 50-70° under a gentle current of air. Add 0.25 ml. of a nitrating reagent prepared by mixing 20 grams of potassium nitrate, 100 ml. of concentrated sulfuric acid, and 10 ml. of water. Heat at 40-44° for 15 minutes and add 0.5 ml. of 15% hydroxylamine hydrochloride solution, followed by 6.25 ml. of 1:3 ammonium hydroxide. Mix after the addition of each reagent and let stand at room temperature for 1 hour. Read at 540 mμ against a reagent blank within 30 minutes.

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[16] S. C. Pan and D. Perlman, *Anal. Chem.* 26, 1432-38 (1954); Haruo Yoshii, *J. Antibiotics (Japan)* Ser. B 8, 387-90 (1955); *Ibid.* 9, 18-22 (1956).

About 0.01 mg. per ml. Acidify a 5-ml. cell-free filtrate of a sample containing 0.01-0.12 mg. of phenylacetic acid with 0.3 ml. of 1:3 sulfuric acid as indicated by a pink to thymol blue indicator. Add 1.5 grams of anhydrous sodium sulfate and shake with 18 ml. of toluene for 30 seconds. Centrifuge for 5 minutes with the tubes loosely stoppered. Add 1 ml. of 1% sodium carbonate solution to 15 ml. of the toluene extract, shake, and repeat the centrifuging. Remove the toluene layer and discard. Add 0.1 ml. of 10% potassium ferricyanide solution. Heat at 100° for 10 minutes. Maintain at 70-80° and pass a gentle air current over the liquid surface for 5 minutes to completely remove any residual toluene. Dilute the residue to 1 ml. with water and develop as for 0.05 mg. of phenylacetic acid per ml., using a 4-ml. aliquot.

PHENOXYACETIC ACID

Upon nitration, phenoxyacetic acid forms a phenolic derivative which appears yellow in ammonium solution.[17] Phenoxyacetic acid is separated from phenoxymethylpenicillin, penicillin V, by extraction with benzene at pH 2 from a saturated ammonium sulfate solution. Penicillin V is converted to phenoxymethylpenicilloic acid with sodium hydroxide and remains in solution. Penicillin V can be determined by difference when the ammonium sulfate solution is extracted with methyl isobutyl ketone in place of benzene. Both penicillin V and phenoxyacetic acid are extracted and the concentration of phenoxyacetic acid, determined previously after benzene extraction, is subtracted from the total reading as a measure of penicillin V. Beer's law is followed for 0.005-0.05 mg. of phenoxyacetic acid. The color is influenced to some extent by the concentration of ammonium hydroxide used to neutralize the acid after nitration. Therefore, standards and unknown solutions should contain the ammonium hydroxide from the same source.

Procedure—*Penicillin fermentation liquor.* Mix a 5-ml. sample with 1 ml. of 8% sodium hydroxide solution at 22° and after 15 minutes, neutralize with 1 ml. of 1:16 sulfuric acid. Dilute to 10 ml. with water. To a 1-ml. aliquot add 1 ml. of 1:35 sulfuric acid and saturate the solution with

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[17] J. Birner, *Anal. Chem.* **31,** 271-3 (1959).

2 grams of dry powdered ammonium sulfate. Mix by swirling. The pH should be approximately 2. Add 5 ml. of benzene and shake vigorously for 45 seconds followed by centrifuging for 5 minutes.

To an aliquot in benzene containing 0.05-0.005 mg. of phenoxyacetic acid per ml., add 1 drop of 0.4% sodium hydroxide solution and evaporate the benzene to dryness at 60-70°. Blow a gentle current of air over the solution to hasten evaporation of the solvent. Add 0.5 ml. of 10% potassium nitrate solution in concentrated sulfuric acid and heat at 100° for 15 minutes. Cool, add 1.5 ml. of water, and mix. Cool in water and gradually add 5 ml. of 1:2 ammonium hydroxide and mix. Read at 415 mμ and correct for the fermentation medium absorption.

2-METHYL-4-CHLOROPHENOXYACETIC ACID

2-Methyl-4-chlorophenoxyacetic acid is determined in the presence of the 2-methyl-6-chloro isomer by direct spectrophotometric measurement in the ultraviolet.[18] The $E_{1\text{cm}}^{1\%}$ for 2-methyl-4-chlorophenoxyacetic acid is 289 at 207 mμ and 485 at 228.5 mμ while that for the 2-methyl-6-chloro isomer is 422 at 216 mμ. By measuring the absorbance at 228.5 mμ and 233 mμ it is possible to determine the compound. Beer's law is followed up to 0.01 mg. per ml. Prior separations are required for commercial insecticide mixtures.

2-Methyl-4-chlorophenoxyacetic acid is measured directly at 280 mμ after chromatography on a column at pH 6.5.[19] Chloroform and acetone are the solvents. The acid is read in methanol.

Procedure—*Phytopharmaceutical products.* Use a sample containing 100 mg. of 2-methyl-4-chlorophenoxyacetic acid in 50 ml. of water and 20 ml. of 1:1 hydrochloric acid. Extract once with a 70-ml. portion of chloroform, twice with 50-ml. portions. Add 50 ml. of a saturated sodium bicarbonate solution to the combined extracts. Shake, remove the chloroform layer, and extract it twice with 40 ml. of half-saturated solution of sodium bicarbonate. Neutralize the combined bicarbonate extracts with 1:1 hydrochloric acid, adding 10 ml. in excess. Extract with 70 ml. of chloroform. Filter the chloroform through a cotton plug and repeat the chloroform extraction twice. Digest the chloroform extracts at 100° until traces of water are eliminated by the formation of a ketone. Cool and dilute to an appropriate volume.

As the buffer prepare a 3.45% solution of monosodium phosphate monohydrate and a 3.55% anhydrous disodium phosphate solution. As a buffer for pH 6.5 mix 44 parts of the first with 56 parts of the second.

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[18] Michel Duvaux, *Chim. & Ind.* (*Paris*) **70,** 981-4 (1953).

[19] J. Henriet, *Parasitica* **11,** 81-102 (1955).

Transfer a 5-ml. aliquot of the sample in chloroform to a 550 × 20 mm. chromatography column containing Hyflo Super-Cel in equilibrium with the buffer for pH 6.5. Elute with a 1:1 mixture of chloroform and acetone, discarding the first 150 ml. Collect the following 75-, 150-, and 50-ml. aliquots. Most of the test substance is in the second. Evaporate the fractions to dryness. Take up with 20 ml. of methanol and read each at 280 mμ.

2,4-Dichlorophenoxyacetic Acid, 2,4-D

Adaptations of the existing chromotropic acid method for 2,4-D acid (Vol. III, p. 337) makes possible the reading of minute quantities of this herbicide.[20] Interferences are removed by chromatographic separation on diatomaceous silica, and any acidic material remaining after this treatment is bleached with stannous chloride in sulfuric acid, which has no effect on the color of the 2,4-D acid complex. The reaction is not specific since formaldehyde and phenoxyacetic acid and other compounds which form formaldehyde also give the color reaction.

Procedure—*Grain or seed.* Shake a 200-gram 10-mesh sample for 1 hour with 500 ml. of chloroform, 500 ml. of ether, and 10 ml. of glacial acetic acid to extract 2,4-D acid. Filter with suction and rinse the extracting flask with 50 ml. of chloroform and 50 ml. of ether. Wash the residue with the rinsing solution. Extract the 2,4-D acid from the filtrate with 250 ml. of 4% sodium hydroxide solution followed by two 200-ml. portions of 1% sodium hydroxide solution. Wash the combined aqueous extracts with 100 ml. of chloroform. Acidify the aqueous portion with 10 ml. of concentrated hydrochloric acid and extract 2,4-D acid with 125 ml. of ether. Repeat the extraction with 100 ml. of ether. Filter the combined ether extracts and extract the 2,3-D acid with 50 ml., 25 ml., and 25 ml. of 2% sodium hydroxide solution. Wash the combined alkaline extracts with three 10-ml. portions of chloroform and discard these washings. Acidify the aqueous layer with 10 ml. of concentrated hydrochloric acid. Add 5 ml. of 40% phosphotungstic acid solution to separate proteins and extract the 2,4-D acid with three 10-ml. portions of chloroform. Combine the chloroform extracts to separate 2,4-D acid from interference by chromatography. Prepare a 1.7 × 50 cm. chromatographic column, one end of which is constricted and attached to a 0.5 × 10 cm. tube. Place a glass wool plug at the constriction and pack diatomaceous silica, Hyflo Super-Cel, into the tube for a length of 20 cm.

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[20] Katsura Munakata, *Japan Analyst* 1, 199-200 (1952); R. P. Marquardt and E. N. Luce, *J. Agr. Food Chem.* 3, 51-3 (1955).

Filter a 30-ml. chloroform aliquot containing up to 0.1 mg. of 2,4-D acid into the column and push into the column with air pressure. Percolate 150 ml. of 3% methanol solution in chloroform through the column at a rate of 150 ml. per hour followed by 25 ml. of 7% methanol solution in chloroform at the same rate. Discard these effluents. Pass 150 ml. of 7% methanol solution in chloroform through the column at the same rate, collecting the effluent.

Prepare a buffered extraction solution at pH 6.85 by dissolving 19 grams of dibasic sodium phosphate heptahydrate and 10 grams of monobasic sodium phosphate monohydrate in water and diluting to 1 liter. Extract the 2,4-D acid from the effluent with three 25-ml. portions of the buffered extraction solution. Wash the combined extracts with three 10-ml. portions of chloroform and acidify with 2 ml. of concentrated hydrochloric acid. Extract the 2,4-D acid with three 10-ml. portions of chloroform. Combine the chloroform extracts and decant from any water globules. Evaporate just to dryness at 100°. Cool and add 5 ml. of 0.1% chromotropic acid solution in concentrated sulfuric acid to the residue. Mix and heat at 150° for exactly 10 minutes. The purple color of 2,4-D acid will develop at this point. Cool to room temperature. To bleach the color of any other acidic material present, pour with stirring into 30 ml. of 5% stannous chloride solution in 1:4 sulfuric acid. Start timing and cool rapidly to room temperature. After a 5-minute interval dilute to 50 ml. with the stannous chloride solution. Mix and filter. After 30-45 minutes, read at 565 mμ against water.

3-INDOLEACETIC ACID

The fluorescence of 3-indoleacetic acid is read after treatment with cupric sulfate in the presence of sulfuric acid. The acid is extracted with ether.[21]

3-Indoleacetic acid is oxidized with ferric chloride (Vol. II, p. 342) in the presence of either perchloric acid or sulfuric acid. In the presence of ascorbic acid the sample should be chromatographed using a 10:1:1 mixture of isopropanol, water, and concentrated ammonium hydroxide as a solvent.[22] 3-Indolecarboxylic acid, 3-indolepropionic acid, 3-indolecarboxy aldehyde, *dl*-tryptophan, indole, and skatole give negligible colors.[23] Hydrogen peroxide and organic peroxides interfere with the determination of small amounts of 3-indoleacetic acid by most methods.[24] The reaction is reversible on removal of the peroxide. Alternative reagents are ferric chloride in glacial acetic acid [25] or in trichloroacetic acid.[26]

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[21] Yu. V. Rakitin and K. L. Povolotskaya, *Fiziol. Rastenii, Akad. Nauk S.S.S.R* **4**, 285-92 (1957).

[22] Federica Trezzi, *Nuovo giorn. bot. ital.* **61**, 391-4 (1954).

Procedure—*Plant material.* To a 2.5-5 gram sample add 0.25-0.5 ml. of 10% acetic acid and 2.5-5 ml. of 95% ethanol. Mix and add 25-50 ml. of diethyl ether. Let stand for 12-18 hours. The alcohol-ether extract contains free 3-indoleacetic acid and the residue contains bound acid. Filter and wash the residue with ether. Put the residue aside for later determination of bound indoleacetic acid.

Combine the washings and filtrate and evaporate the ether. Add 0.3-0.5 gram of sodium bicarbonate and take up in a saturated sodium bicarbonate solution. The total volume at this point should be 20-25 ml. of aqueous solution at pH 7.8-8.0. Extract with ether to remove fats and other ether-soluble substances. Discard this extract. Adjust the pH of the bicarbonate solution to 4-4.5 with 20% acetic acid and extract the 3-indoleacetic acid with ether. Evaporate the ether and heat the residue with solid sodium bicarbonate. Take up in a saturated sodium bicarbonate solution and re-extract this alkaline solution with ether. Discard these washings and adjust the pH of the aqueous phase to 4.0-4.5 with 20% acetic acid. Evaporate any dissolved ether and dilute to 25 ml. Extract a 10-12 ml. aliquot with three portions of ether to remove any remaining 3-indoleacetic acid. Evaporate the ether from the aqueous layer and use a portion as a blank.

Dilute an aliquot of the aqueous alkaline layer containing the 3-indoleacetic acid to 6 ml. Add 1 ml. of 1.25% cupric sulfate pentahydrate solution and 3 ml. of concentrated sulfuric acid. Heat at 100° for 5 minutes, chill rapidly, and add 10 ml. of water. Read the fluorescence.

To determine the bound 3-indoleacetic acid in the residue left after the original filtration, remove any ether present by evaporation. Hydrolyze by refluxing for 3 hours with a 3% ethanolic alkali solution using 6 ml. of alkali solution for each gram of the original sample. Cool, adjust the pH to 5.5-5.8 with 20% acetic acid, and evaporate to 6-8 ml. Cool and add 25-50 ml. of ether. Extract for 12-18 hours. Separate this ether layer and evaporate to dryness. Continue as for free 3-indoleacetic acid from "Add 0.3-0.5 gram of sodium bicarbonate and take up in a saturated sodium bicarbonate solution."

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[23] Paul Emil Pilet, *Rev. gén. botan.* **64,** 106-22 (1957).

[24] S. M. Siegel and R. L. Weintraub, *Physiol. Plantarum* **5,** 241-7 (1952).

[25] Ichiro Tezuka, *Siekagaku* **29,** 55-7 (1957).

[26] A. M. Meyer, *Nature* **182,** 1670-1 (1958).

By ferric chloride. As a reagent mix 3 ml. of 24% ferric chloride solution, 60 ml. of concentrated sulfuric acid, and 100 ml. of water. To 2 ml. of sample containing 0.00025-0.01 mg. per ml. of 3-indoleacetic acid add 8 ml. of reagent and maintain at 40° for 12-20 minutes in the dark. Cool and read at 535 mμ.

5-HYDROXYINDOLEACETIC ACID

Nitrous acid is a reagent for 5-hydroxyindoleacetic acid. Dinitrophenylhydrazine is the complexing agent for interfering keto acids. Chloroform is used to extract the 5-hydroxyindoleacetic acid from the hydrazones.[27] The color is read at pH 7 since it is unstable at a higher pH. The ether used must be peroxide-free.

5-Hydroxyindoleacetic acid is also determined with *p*-dimethylaminobenzaldehyde.[28] Beer's law is followed for 0.002-0.025 mg. of 5-hydroxyindoleacetic acid per ml. at 600 mμ. Solutions adjusted to pH 7 show maximum absorption at 221 mμ and follow Beer's law in the range of 0.001-0.015 mg. per ml.

Procedure—*Urine. By nitroso-2-naphthol.* To a 6-ml. sample containing 0.01-0.2 mg. of 5-hydroxyindoleacetic acid, add 6 ml. of 0.5% 2,4-dinitrophenylhydrazine solution in 1:5 hydrochloric acid. If a larg precipitate of the hydrazones of α-keto acids forms, centrifuge. After 3 minutes extract the 5-hydroxyindoleacetic acid with 25 ml. of chloroform Centrifuge. Add a 10-ml. aliquot of the aqueous layer to 25 ml. of ethe and 4 grams of sodium chloride. Shake for 5 minutes and centrifuge a 20-ml. aliquot of the ether layer. Add to 1.5 ml. of a 0.5 *M* phosphat buffer for pH 7. Shake for 5 minutes and centrifuge. Remove the ethe layer.

Add a 1-ml. aliquot of the aqueous layer containing 0.01-0.2 mg. o 5-hydroxyindoleacetic acid buffered at pH 7 to 0.5 ml. of 0.1% 1-nitroso-2-naphthol solution in ethanol. Add 0.5 ml. of fresh nitrous acid reagen prepared by mixing 0.2 ml. of 2.5% sodium nitrite solution with 5 ml. o 1:16 sulfuric acid. Mix and heat at 37° for 5 minutes. Add 5 ml. of eth acetate and shake. Remove the ethyl acetate layer and discard. Repeat with another 5-ml. portion of ethyl acetate. Read the aqueous layer at 540 mμ.

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[27] S. Udenfriend, E. Titus, and J. Weissbach, *J. Biol. Chem.* **216**, 499-505 (1955) A. Carcasona, J. Cervós-Navarro, and F. Unterharnscheidt, *Klin. Wochschr.* 37 253-4 (1959).

[28] P. Correale, *Experientia* **11**, 315-6 (1955).

By p-*dimethylaminobenzaldehyde.* To each volume of 5-hydroxyindoleacetic acid in pure solutions or from aqueous eluates from paper chromatograms, add one-half volume of 2% *p*-dimethylaminobenzaldehyde in concentrated hydrochloric acid. Heat at 56-58° for 10-12 hours and read at 600 mμ.

METHYL METHACRYLATE

A photometric method for methyl methacrylate involves treatment with potassium bromide and bromate, acidification, and addition of iodide.[29]

Procedure—*Air.* Draw a 5-10 liter sample at the rate of 20 liters per hour through 20 ml. of 95% ethanol in 2 absorption flasks. Mix and dilute to 20 ml. Mix a 5-ml. aliquot with 2 ml. of a solution of 0.04% potassium bromide and 0.01% sodium bromate. Acidify with 2 ml. of 1:1 hydrochloric acid. After 10 minutes add 5 drops of 5% potassium iodide solution and read the color.

LACTIC ACID

After dehydration to acetaldehyde, micro quantities of lactic acid in the range of 0.000025-0.00025 mg. in 0.025 ml. are estimated with *p*-hydroxydiphenyl[30] (Vol. III, p. 348). A major source of error is the contamination of reagents, standards, and glassware, and the dispersion of the reagent with the sample. The blank value is decreased by heating the reagent and water for 1 minute at 100°. The reagent on standing gives a higher blank value and should be discarded when the absorption with water becomes higher than 0.17.

Samples are deproteinized with copper sulfate and calcium hydroxide which also serves to remove interferences from ascorbic acid, glucuronic acid, and oxalacetic acid. Copper as copper sulfate is added to the sulfuric acid dehydrating reagent to enhance the color. The concentration of the reagent affects the color development. If the tubes are left standing at room temperature for 30 minutes after addition of reagent and before incubation, the optical density increases. This is avoided by maintaining the tubes in an ice bath.

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[29] N. L. Nemirovskii and G. I. Meerovich, *Gigiena i. Sanit.* **23,** No. 2, 83-5 (1958).

[30] Sjoerd L. Bonting, *Arch. Biochem. and Biophys.* **56,** 307-17 (1955); Z. Duda, *Izvest. Vysshikh Ucheb. Zavedenii. Pishchevaya Tekhnol.* **1959,** No. 1, 141-4.

Choline, α-ketoglutaric acid, malic acid, and pyruvic acid interfere. There is no interference from aconitic acid, adenine, alanine, citric acid, fumaric acid, glucose, glutathione, glycine, *d*-ribose, succinic acid, and urea under the conditions of the method. Beer's law is followed up to 0.01 mg. per ml. The absorption maximum is at 572 mμ. The color is stable for 3 hours.

The red color produced between lactic acid as acetaldehyde and veratrol (Vol. III, p. 348) is converted to a stable green color by adding ferric chloride to the sulfuric acid and heating 4-8 minutes at 100° during the dehydration step.[31] The color obeys Beer's law. Formaldehyde and methylglyoxal interfere. Addition of 2,4-dinitrophenylhydrazine eliminates the interference of methylglyoxal. For this method, carbohydrates are eliminated by treating the deproteinized filtrate with copper sulfate solution, neutralizing, and adding calcium hydroxide.[32] Thiosemicarbazide is also a reagent for lactic acid after conversion to acetaldehyde.[33]

The determination of lactic acid after dehydration to pyruvic acid with lactic acid dehydrogenase is accomplished with ferricyanide, reading the resulting ferrocyanide color directly at 405 mμ.[34] The limit of detection in a 1-ml. sample is 0.001 mg. Glyceric acid also reacts with lactic acid dehydrogenase but does not interfere. Lactic acid treated with an alkaline solution of iodine and potassium iodide is converted to iodoform for extraction with chloroform and reading in the ultraviolet.[35]

Procedure—*By* p-*hydroxydiphenyl. Biological samples.* To remove proteins and other interfering substances treat 1 volume of the sample with 5 or more volumes of 2% copper sulfate pentahydrate solution and add 10 mg. of calcium hydroxide for each 0.1 ml. of total volume. Shake and let stand at room temperature for 30 minutes. Centrifuge for 1 minute.

As a reagent, dissolve 150 mg. of *p*-hydroxydiphenyl in 2.5 ml. of 4% sodium hydroxide solution with heating. Slowly add 17.5 ml. of water and mix. To a 0.025-ml. sample containing 0.000025-0.00025 mg. of lactic acid, add 0.15 ml. of sulfuric acid-copper sulfate reagent prepared by mixing 1 ml. of 2% copper sulfate pentahydrate solution with 60 ml. of concentrated sulfuric acid. Mix and place in an ice bath. Stopper and heat for 15 minutes at 100°. Cool briefly in ice and centrifuge for 1 minute to force down any liquid adhering to the side. Add 0.005 ml. of the *p*-hydroxydiphenyl reagent and shake to disperse the precipitated reagent. Place in ice and stopper. Maintain at 30° for 30 minutes to develop the color and heat at 100° for 1 minute to decompose excess reagent. Cool and read at 570 mμ against water.

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[31] Fritz Kubowitz, *Z. ges. inn. Med. u. Grenzgebiete* 7, 865-82 (1952).

[32] V. Láhn, *Prakt, problémy lékař. chem., I. sjezd lékař, biochemiků Pilsen* 1955, 144-8.

[33] H. J. Starck, *Klin. Wochschr.* 34, 153-5 (1956).

[34] H. J. Holhorst, *Biochem. Z.* 328, 509-21 (1957); Otto Wieland, *Ibid.* 329, 568-76 (1958).

[35] G. Di Modica and E. Angeletti, *Ricerca sci.* 28, 1485-8 (1958).

By potassium ferricyanide. Biological samples. Deproteinize a 1-ml. sample with 2 ml. of 3% perchloric acid solution. Centrifuge and neutralize the supernatant liquid with 5.6% potassium hydroxide solution. Dilute to 3 ml. Place 1 ml. of neutralized, deproteinized sample in an ice bath for 15 minutes. Add to 1 ml. of 0.07 *M* pyrophosphate buffer at pH 8 and 0.15 ml. of 0.3% potassium ferricyanide solution. Dilute to 2 ml. and add 0.01 ml. of a solution of lactic acid dehydrogenase. After 10-15 minutes at 30° read the ferrocyanide color at 405 mμ.

By thiosemicarbazide. Mix a 0.5-ml. sample with 5 ml. of 3.3% perchloric acid and centrifuge. Incubate 1 ml. of the supernatant liquid for 30 minutes at 50° in the outer chamber of a diffusion vessel to remove volatile interfering compounds such as ethanol and acetone. Add 5 ml. of 10% ceric sulfate solution in 1:35 sulfuric acid to the sample and 5 ml. of 0.009% thiosemicarbazide solution in 1:350 sulfuric acid to the inner chamber of the diffusion vessel. Incubate for 3.5 hours at 30° and read the content of the inner chamber at 261.5 mμ or 366 mμ.

ACETOACETIC ACID, DIACETIC ACID

Acetoacetate reacts with diazotized *p*-nitroaniline (Vol. III, p. 352) at pH 5.2 to form *N,N′*-bis(*p*-nitrophenyl)-*C*-acetylformazan which is extracted with ethyl acetate.[36] The reaction is stopped by addition of hydrochloric acid. Pyruvic acid does not interfere. Oxalacetic acid, malonic acid, and *l*-ascorbic acid give appreciable color reactions but only *l*-ascorbic acid interferes with the color reaction of acetoacetate.

After distillation into sodium bisulfite solution either acetone acetoacetic acid are determined by vanillin in alkaline solution,[37] page 277.

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[36] P. G. Walker, *Biochem. J.* 58, 699-704 (1954).

[37] V. E. Levine and M. Taterka, *Clin. Chem.* 3, 624-30 (1957).

Procedure—*By diazotized* p-*nitroaniline.* To prepare the diazotized *p*-nitroaniline, add 3 ml. of 0.5% sodium nitrite solution to 20 ml. of 0.05% *p*-nitroaniline solution in 1:240 hydrochloric acid. Add 7 ml. of 1.6% sodium acetate solution so that the final pH is 4.

To a 0.5-ml. neutral sample containing 0.001-0.01 mg. of acetoacetate add 0.5 ml. of 8.2% sodium acetate-6% acetic acid buffer at pH 5.2, and 3 ml. of diazotized *p*-nitroaniline. Let stand for 30 minutes at room temperature. To stop the color reaction add 1 ml. of 1:2 hydrochloric acid and extract with 4 ml. of ethyl acetate. Read the ethyl acetate layer at 440 mμ.

Ethyl Acetoacetate

A rapid method for determining microgram quantities of ethyl acetoacetate depends on the ultraviolet absorption of the ethyl acetoacetate carbanion formed by the addition of sodium hydroxide.[38] Excess sodium hydroxide maintains the maximum absorbance at 272.5 mμ. Once this concentration is reached additional sodium hydroxide will not prolong the life of the color. Since the carbanion is not stable, a constant time after addition of the sodium hydroxide should be established for reading.

Procedure—To a 5-ml. sample containing 0.001-0.007 mg. of ethyl acetoacetate add 1 ml. of 0.4% sodium hydroxide solution and mix. Read at 272.5 mμ against a reagent blank after a standardized time.

Glyceric Acid and Phosphorylated Glyceric Acid, Dihydroxypropionic Acid

Glyceric acid and naphthoresorcinol react in the presence of concentrated sulfuric acid to give a blue color. The same reaction is given by the mono- or diphosphate esters.[39] The material must be dry, otherwise the color becomes greenish. Appropriate amounts are 0.15-0.4 mg. as glyceric acid.

The same reaction is given by 2,7-dihydroxynaphthalene and by chromotropic acid. The latter is the most sensitive.[40] At equivalent concentrations there is less than 5% interference by glucose, fructose, their 1- and 6- mono and diphosphates, sedoheptulose, ribose, erythrose, glyceraldehyde, dihydroxyacetone, lactic acid, pyruvic acid, glycolic acid, acetic acid, glycerol, formaldehyde, and adenosine mono-, di-, and triphosphates.

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[38] Francis N. McMillan, *Anal. Chem.* 28, 1532-3 (1956).

[39] S. Rappoport, *Biochem. Z.* **289,** 406-10 (1937).

[40] Grant R. Bartlett, *J. Biol. Chem.* **234,** 469-71 (1959).

By chromatographic separation the glycerates can be separately determined.

Procedure—*By naphthoresorcinol. Blood.* Mix 5 ml. of blood with 5 ml. of 20% trichloroacetic acid and 15 ml. of water and filter. Neutralize 15 ml. to an orange color with methyl orange using 30% sodium hydroxide solution and finally 4% sodium hydroxide. Add 2 ml. of saturated lead acetate solution and 10 ml. of 96% ethanol to complete precipitation of the phosphoglycerate. After 1 day centrifuge. Wash the precipitate with 3 ml. of water. Suspend the precipitate in 2 ml. of water and pass hydrogen sulfide through for 15 minutes. Filter, wash the precipitate with water, and dilute to 10 ml. Transfer 3 ml. for human blood or 2 ml. for porcine blood to a Wassermann Tube, add 2 drops of concentrated hydrochloric acid, and evaporate to dryness within 1 hour at 100°. Add 2 drops of concentrated hydrochloric acid and again take to dryness within 30 minutes.

Add 2 ml. of fresh 0.1% solution of naphthoresorcinol in concentrated sulfuric acid and heat at 100° for 1 hour. Dilute to 25 ml. with concentrated sulfuric acid and read at 700 mμ.

By 2,7-dihydroxynaphthalene. Mix 2 ml. of prepared solution with 5.8 ml. of fresh 0.01% solution of hydroxynaphthalene in concentrated sulfuric acid. Heat at 100° for 30 minutes for glyceric acid, 60 minutes for the monophosphate ester, 120 minutes for the diphosphate ester. Read at 480 mμ.

By chromotropic acid. Proceed with this reagent replacing 2,7-dihydroxynaphthalene but heat 30 minutes for glyceric acid or the monoester, 120 minutes for the diester. Read at 700 mμ.

α-(4-Chloro-2-methylphenoxy) propionic Acid

The weed killer, α-(4-chloro-2-methylphenoxy)propionic acid, can be expected to contain α-(6-chloro-2-methyl)propionic acid, α-(4,6-dichloro-2-methyl)propionic acid, and α-(2-methylphenoxy)propionic acids as well as possibly β isomers. Since the ratio of *d* and *l* isomers of the agent is constant the acid can be determined although only one isomer is an effective herbicide. After separation by liquid-liquid chromatography the α-(4-chloro-) acid is read in the ultraviolet.[41]

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[41] L. A. Haddock and L. G. Phillips, *Analyst* **84**, 94-101 (1959).

Procedure—As a buffer for pH 7.3 mix 165 ml. of 3.725% solution of anhydrous disodium phosphate with 35 ml. of 3.45% solution of monosodium phosphate monohydrate. Triturate 25 grams of Hyflo Super-Cel with 12.5 ml. of this buffer. Mix equal volumes of diethyl ether and chloroform and protect from strong light. To equilibrate shake 1 liter of this solvent mixture with 50 ml. of the buffer for pH 7.3. Cool to room temperature and filter.

Triturate the buffer-Super-Cel mixture with 250 ml. of the equilibrated solvent. Place a cotton plug in the bottom of a 50 × 1.6 cm. chromatographic column and add solvent to fill the tube 2/3 full. Add the slurry and pack the first 0.5 cm. Pack the balance of the column in 1-2 cm. sec-

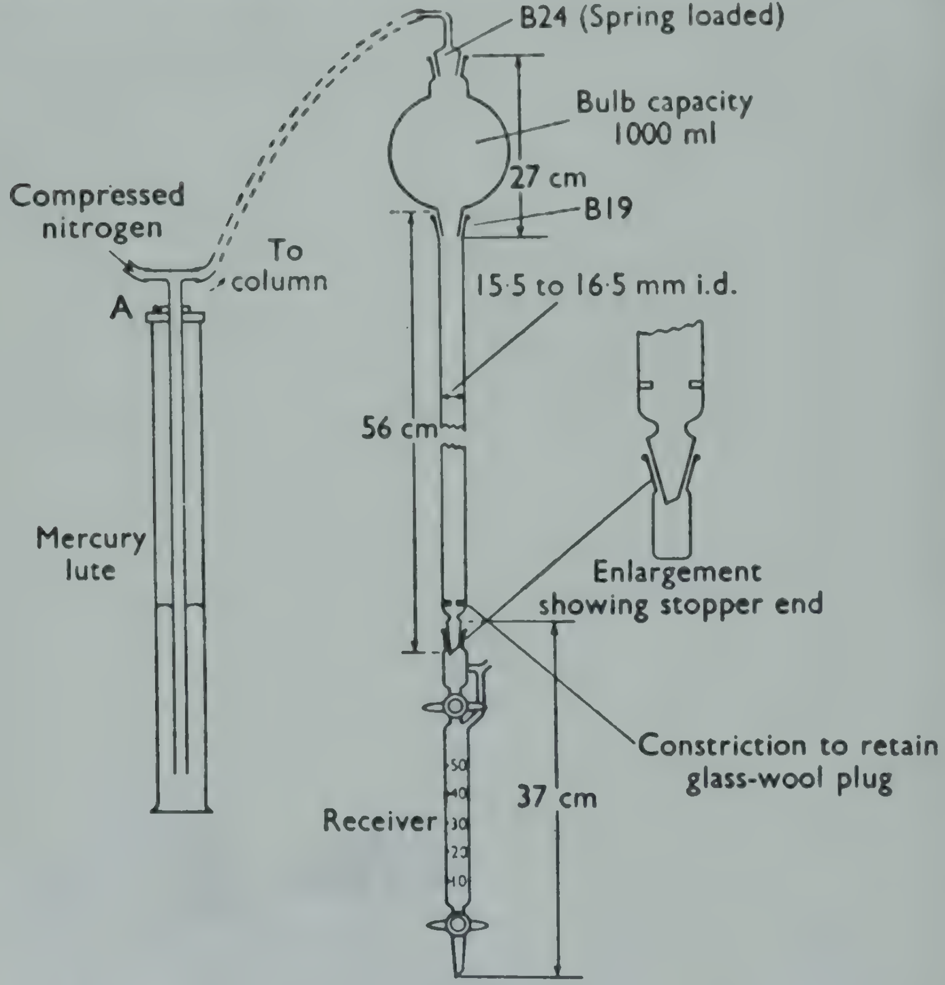
B24 (Spring loaded)
Bulb capacity 1000 ml
27 cm
B19
Compressed nitrogen
To column
A
15·5 to 16·5 mm i.d.
56 cm
Mercury lute
Enlargement showing stopper end
Constriction to retain glass-wool plug
37 cm
Receiver
50
40
30
20
10

FIG. 16

Column assembly for determination of α-(4-chloro-2-methylphenoxy)propionic acid ions avoiding entrapment of air. When finished there should be 20-30 ml. of solvent above the column. Assemble the apparatus shown in Figure 16. At 3-5 lb. per sq. inch the flow rate should be 2-2.5 ml. per minute. Lower the solvent level to close to the packed column.

Dissolve 0.1 gram of 4,6-dichloro-2-methylphenoxypropionic acid, 0.5 gram of 4-chloro-2-methylphenoxypropionic acid, and 0.2 gram each of 6-chloro-2-methylphenoxypropionic acid and 2-methylphenoxypropionic acid in 100 ml. of the ether-chloroform solvent. Remove the separatory funnel and place 1 ml. of this on the surface of the column. Force through by nitrogen pressure until just absorbed and follow successively with two 1-ml. portions of the equilibrated solvent mixture. Replace the separatory funnel filled with the equilibrated solvent and pass about 1 liter through the column. Collect successive 10-ml. portions of the eluate. Add 3-4 ml. of water to each and evaporate until the ether-chloroform is removed. Titrate each with carbon dioxide-free 0.04% sodium hydroxide solution passing nitrogen or carbon dioxide-free air through the solution during titration. This should pass through for at least 2 minutes before titration to remove dissolved carbon dioxide. Plot the volumes against the titrations to be sure that the troughs between the peaks are well defined. If not the column has not been properly packed. Do not let the column become dry and if set aside for longer than 2 days flush with 50 ml. of freshly equilibrated solvent.

Take up 0.4 gram of the sample of acids and dilute to 50 ml. with diethyl ether. Pass 50 ml. of equilibrated solvent through the column stopping at the level of the Super-Cel. Add 1 ml. of sample solution to the column and pass into the column. Do the same with two 1-ml. portions of equilibrated solvent. Elute the column with about 1 liter of equilibrated solvent to be sure of completely removing the 3-methylphenoxypropionic acid. Collect the first 30 ml. and the second 30 ml. Then collect the main band (M) of 200 ml. as determined by the calibration. This will usually be about 120-320 ml. Collect and titrate a 10-ml. aliquot just before the main band to be sure that the main band includes all the 4-chloro acid. Collect the next 100 ml. after the main band (F).

As standard dissolve 0.2 gram of 4-chloro-2-methylphenoxypropionic acid in 100 ml. of diethyl ether. Dilute 1 ml. of this to 50 ml. (G) with the fraction (F) collected after the main band. Read at 287 mμ F against the first fraction of eluate as x, G against F as z, and G against M as h.

The % α-(4-chloro-2-methylphenoxy)propionic acid

$$= \frac{z + x/3 - h}{5z} \times \frac{\text{volume of } M \text{ in ml.}}{\text{weight of sample in grams}}.$$

Glyoxylic Acid, Glyoxalic Acid

The oxidation of glyoxylic acid phenylhydrazone with potassium ferricyanide in the presence of excess phenylhydrazine[42] produces an intense red compound, 1,5-diphenylformazancarboxylic acid. The method is not specific for glyoxylic acid as aldehydes give a similar reaction.

The color is stable for 2 hours at pH 1.2 or below while variations in the pH cause unstability or precipitation. Hydrogen peroxide cannot be used as an oxidizing agent since it produces unstable colors. Heating the glyoxylic acid phenylhydrazone at 110° for more than 7 minutes results in unreproducible color densities. The phenylhydrazine reagent should be fresh and glyoxylic acid stock solutions should be kept under nitrogen and refrigerated as they are oxidized in air. Beer's law is followed. Formates and aldehydes interfere. Methanol, ethanol, phenol, acetic acid, oxalic acid, glycolic acid, pyruvic acid, acetone, urea, acetophenone, glucose, sucrose, aniline, chlorobenzene, nitrobenzene, ethyl acetate, methyl propionate, and methylal do not interfere.

Procedure—Incubate a 2.5 ml. sample containing 0.00025-0.025 mg. of glyoxylic acid with 2.5 ml. of 1% phenylhydrazine solution at 110° for 5 minutes. Cool to room temperature and add 2.5 ml. of concentrated hydrochloric acid and 2.5 ml. of 1% potassium ferricyanide solution. Mix well. Read after 2 minutes at 520 mμ against a reagent blank containing 2.5 ml. of water.

α-Ketopropionic Acid, Pyruvic Acid

Pyruvic acid converted to its nitrophenyl hydrazone remains the most common method for its determination (Vol. III, pp. 353-7). However two major modifications have been included: elimination of the deproteinization of the sample, and elimination of the sodium hydroxide to form the red color.[43]

Since the protein is precipitated by the reagent and separated by centrifuging, it is not necessary to deproteinize the sample. By elimination of the sodium hydroxide, the yellow color of the nitrophenyl hydrazone i measured directly. In this way a more stable color is obtained, the sensitivity of the method is increased, and the blank absorbance is less. Although most α-keto acids are determined by this method, a quantitativ distinction between pyruvic acid and either α-ketoglutaric or oxalacetic acid is made possible by varying the reaction times and organic solvent.

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[42] D. N. Kramer, N. Klein, and R. A. Baselice, *Anal. Chem.* **31**, 250-2 (1959).
[43] S. L. Bonting and S. Bonting, *Arch. Biochem. and Biophys.* **58**, 100-8 (1955).

In the separation of pyruvic acid from blood and other α-keto acids by paper chromatography as the nitrophenyl hydrazones, two isomers are found present.[44]

Pyruvic acid is separated from α-ketoglutaric acid by filter paper electrophoresis after isolation as the nitrophenyl hydrazone.[45] Under these conditions the pyruvic acid does not separate into its isomers. The method is much more rapid than chromatography. Acetoacetic acid, which forms a colored hydrazone with the reagent, is removed by heating the protein-free sample for 5 minutes at 100°.

Salicylaldehyde is another reagent for pyruvic acid.[46] All compounds containing a CH_3CO- group linked directly to a hydrogen or carbon atom give a positive reaction with salicylaldehyde. Thus, acetoaldehyde, acetone, diacetyl, levulinic acid, and acetoacetic acid interfere while formaldehyde, oxalacetic acid, ketobutyric acid, and α-ketoglutaric acid do not interfere. Bisulfite ions and formaldehyde in large amounts prevent the development of the color to some extent. Ethanol affects the consistency of the readings. Beer's law is followed and the color remains constant for 1 hour following the 2-hour development period.

Procedure—*By 2,4-dinitrophenylhydrazine.*[47] *Pyruvic acid.* Prepare the reagent by dissolving 100 mg. of 2,4-dinitrophenylhydrazine in 100 ml. of 1:2 hydrochloric acid and dilute with 100 ml. of water. To a 0.02-mg. sample containing 0.00001-0.0001 mg. of α-keto acid add 0.020 ml. of reagent. Mix and centrifuge briefly. Let stand for 5 minutes at 20° and add 0.10 ml. of xylene. Extract hydrazones and excess reagent from the water layer by shaking for 15 seconds. Centrifuge for 1 minute to separate the layers. To a 0.050-ml. aliquot add 0.10 ml. of 10% sodium carbonate solution, holding the pipet tip close to the surface of the liquid so that the carbonate flows below the organic layer. Shake for 30 seconds and centrifuge to separate the layers. Remove the organic layer and read the carbonate layer at 380 mμ against water.

Total α-keto acids. Follow the procedure by 2,4-dinitrophenylhydrazine using ethyl acetate as the solvent in place of xylene, and extending the reaction time with the reagent to 30 minutes at 25°.

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[44] S. Markees and F. Gey, *Helv. Physiol. et Pharmacol. Acta* **11,** 49-54 (1953); S. Markees, *Experientia* **11,** 205-8 (1955).

[45] Henry Tauber, *Anal. Chem.* **27,** 287-9 (1955).

[46] Svante Berntsson, *Ibid.* **27,** 1659-60 (1955).

[47] S. L. Bonting and S. Bonting, *Arch. Biochem. and Biophys.* **58,** 100-8 (1955).

Blood. By paper chromatography.[48] Treat a 4-ml. sample containing heparin with 20 ml. of 10% trichloroacetic acid and centrifuge. Heat the supernatant liquid for 5 minutes at 100° and cool to 20°.

Mix 15 ml. of the protein-free sample with 2.5 ml. of 0.2% 2,4-dinitrophenylhydrazine solution and let react for 5 minutes. Extract with 15 ml. of toluene for 1 minute and wash the extract with a small portion of water. Remove traces of water by filtration and evaporate 12 ml. of the toluene extract to dryness *in vacuo* at 30°. Dissolve the residue in 4 ml of 10% trichloroacetic acid solution and apply 1 drop to filter paper. Effect ascending chromatography with 3% ammonium hydroxide solution saturated with butanol for 14 hours. Dry at 20°. Cut out the 2 spots developed in 3 × 4 cm. pieces and shred. Elute each with 8 ml. of 10% sodium carbonate solution by shaking for 2 hours at 20° in the dark. Filte and add 1.5 ml. of 8% sodium hydroxide solution to 6 ml. of each filtrate Read after 2 minutes. The spots are 2 isomers and results are about 20% higher than by other methods.

By filter-paper electrophoresis.[49] *Blood.* Add a 15-ml. fresh sampl immediately to 105 ml. of water and 15 ml. of 1:53 sulfuric acid. Mix an add 15 ml. of 10% sodium tungstate solution. Shake for 1 minute, l stand for 10 minutes, and centrifuge for 7 minutes in a refrigerated centrifuge at 5°.

To prepare the reagent dissolve 400 mg. of 2,4-dinitrophenylhydrazi in 40 ml. of 95% ethanol and 10 ml. of concentrated hydrochloric aci Dilute to 200 ml. with 95% ethanol. To a 100-ml. protein-free samp containing 0.1-1.5 mg. of keto acid or a mixture of keto acids, add 5 m of 2,4-dinitrophenylhydrazine reagent. Mix and let stand for 30 minut at 25°. Extract the hydrazones with three 30-ml. portions of ethyl acetat If emulsions form, separate the layers by centrifuging. Extract the combined ethyl acetate layers with two 30-ml. portions of 10% sodium ca bonate solution. Wash the combined sodium carbonate layers with tv 20-ml. portions of ethyl acetate. Cool the sodium carbonate layers a acidify with 20 ml. of cold 1:1 hydrochloric acid, added dropwise wh rotating the flask to eliminate the formation of isomers or polymers in t presence of excess acid.

Extract the hydrazones with two 20-ml. portions of ether. Dry t combined ether extracts with anhydrous sodium sulfate. Decant and wa the sodium sulfate with 2 ml. of ether. Add the washing to the extract a evaporate the ether at 100°, removing final traces of ether *in vacuo.* Dissolve the dry hydrazones from a blood sample in 0.1 ml. of 95% ethanol and from other samples in 0.2-0.4 ml. of 95% ethanol.

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[48] S. Markees and F. Gey, *Helv. Physiol. et Pharmacol. Acta* **11**, 49-54 (1953).
[49] Henry Tauber, *Anal. Chem.* **27**, 287-9 (1955).

Prepare a barbiturate buffer at pH 8.45 by dissolving 5.58 grams of sodium barbital and 3.88 grams of sodium acetate trihydrate, in 590 ml. of water. Add 4.8 ml. of 1:10 hydrochloric acid to bring the pH to 8.5 and dilute to 600 ml. with water. Saturate 285 × 65 mm. filter paper strips with the barbiturate buffer and remove excess buffer by blotting with filter paper. Apply 0.10 ml. of the hydrazones on a line at 80 mm. from one end of the paper. Conduct the electrophoresis for 2-3 hours for pure hydrazones and 4-5 hours for hydrazones from blood samples. Remove the frame containing the strips and dry at 40°.

The hydrazones appear dark red-brown or dark brown. Cut out the bands including the yellow center line with the α-ketoglutaric acid section. Slice, and add 6 ml. of 4% sodium hydroxide solution to the α-ketoglutaric acid hydrazone shreds, and 12 ml. of 4% sodium hydroxide solution to the pyruvic acid hydrazone shreds or 24 ml. if the blood sample was used. Stopper and shake for 2 minutes, followed by centrifuging for 10 minutes. If filter fibers are present, recentrifuge. Read at once.

By salicylaldehyde. To a sample containing up to 0.09 mg. of pyruvic acid, add 5 ml. of 25% sodium hydroxide solution and dilute to 35 ml. with water. Add 0.5 ml. of salicylaldehyde and shake for a few minutes. Add 10 ml. of 25% sodium hydroxide solution and dilute to 50 ml. with water. Read after 2 hours at 456 mμ against a reagent blank.

5-Ketogluconic Acid and 5-Ketogluconate

5-Ketogluconate reacts with 1-methyl-1-phenylhydrazine sulfate at 98° and pH 3-4 to yield a rose colored osazone with an absorption peak at 350 mμ. The reaction is relatively specific and can be used to measure quantities as small as 0.01 mg. in the presence of aldoses, ketohexoses, and 2-ketogluconate.[50] Treatment with iodine to remove aldoses lowers the absorbance of the product formed by about 20%. If this step is included, standards used for the calibration curve must therefore be similarly treated.

Under the conditions of the procedure, 2-ketogluconate shows no peak and little absorption at 350 mμ. If a large excess of 2-ketogluconate is present, the value for 5-ketogluconate is too high and is corrected after determining the amount of 2-ketogluconate present. Absorbance of the product formed by 2-ketogluconate with the reagent is 4% that of 5-ketogluconate. Dihydroxyacetone interferes, while glucose, gluconate, and fructose do not. Although acetate has little effect on the reagent, the absorbance of the product formed with glucose and 2-ketogluconate with the reagent is higher in the presence of acetate. Beer's law is followed over the range of 0.005-0.5 micromole of 5-ketogluconate.

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[50] Michael Schramm, *Ibid.* **28**, 963-5 (1956).

A spectrophotometric method is based on the reduction of an arsenomolybdate reagent at 50°.[51] At 100° the reagent is reduced by sugars but at 50° there is no reduction by glucose, mannose, sorbose, galactose, maltose, gluconic acid, glucuronic acid, or galacturonic acid in concentrations up to 0.8 mg. At temperatures over 70° aldohexoses reduce the reagent.

2-Ketogluconate, fructose, and sorbose give interfering color. If present in 5 times the amount of acid, fructose and sorbose are separated from the acid by an anion-exchange resin on which the acid is retained. The ketogluconic acid is then eluted with 5 times the volume of the resin of 1:500 sulfuric acid. The method is specific for as little as 0.005 mg. of acid and Beer's law is followed.

Procedure—To remove proteins, add 70% perchloric acid to the sample to a final concentration of 12%. Centrifuge to clear. If no aldoses are present skip to "Recrystallize 1-methyl-1-phenylhydrazine sulfate. . . ." To remove aldoses, adjust the deproteinized sample to pH 7 with 12% sodium hydroxide solution and dilute to the desired volume. Prepare a buffer for pH 10 by dissolving 4.2 grams of sodium bicarbonate and 36 grams of sodium carbonate in 1 liter of water. To a 2.5-ml. aliquot containing at least 0.08 mg. of 5-ketogluconate and up to 20 micromoles of aldose, add 1 ml. of the carbonate buffer. Add 0.8 ml. of 1.27% solution of iodine in 2.5% potassium iodide solution. Shake, stopper, and let stand in the dark for 30 minutes. To decolorize the iodine, add 0.2 ml. of 4.2% sodium bisulfite solution and shake, adding additiona bisulfite solution if necessary. Add 1 ml. of 1:5 phosphoric acid and shak to expel carbon dioxide.

Recrystallize 1-methyl-1-phenylhydrazine sulfate by addition of ho alcohol to a hot aqueous solution. Remove interfering substances by shaking the crystals with hot alcohol. The purified product should give colorless reagent. Adjust a 2-ml. aliquot of prepared sample containin 0.01-0.1 mg. of 5-ketogluconate to pH 3-4 with 12% sodium hydroxid solution or 1:12 hydrochloric acid. Heat with 1 ml. of 2% 1-methyl-1 phenylhydrazine sulfate solution at 100° for 40 minutes and cool to room temperature. Read at 350 mμ against a reagent blank.

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[51] N. Nelson, *J. Biol. Chem.* **153,** 375-80 (1944).

By arsenomolybdate. As a reagent, dissolve 25 grams of ammonium molybdate in 450 ml. of water and add 20 ml. of concentrated sulfuric acid. Mix and add 3 grams of sodium arsenate heptahydrate dissolved in 25 ml. of water. Mix, and incubate at 37° for 24-28 hours or stir at 55° for 25 minutes. Dilute an aliquot containing 0.005-0.08 mg. of 5-keto-*d*-gluconic acid to 1 ml. with water and add 5 ml. of reagent which has been diluted 1:3 with water. Heat at 50° for 2 hours and read at 660 mμ.

PHENYLPYRUVIC ACID

The defect of the unbuffered ferric chloride reaction for phenylpyruvic acid, Vol. III, p. 357, has been that the maximum starts to fade after being reached in about 3 minutes. Use of ferric alum,[52] or ferric citrate[53] gave reduced sensitivity. Buffered ferric chloride is more sensitive[54] at a reduced temperature but still reaches a maximum at 10° in 6 minutes. This is followed by relatively rapid decay.

By use of a glycine buffer containing uranium, low temperature, ferrous ammonium sulfate as stabilizer, and storage in darkness the reaction is appropriate for use.[55] Phenylacetic and phenyllactic acids are tolerated. Beer's law is followed up to 0.8 mg. per ml.

Procedure—To prepare a glycine buffer at pH 2.2, dissolve 4.35 grams of glycine and 3.4 grams of sodium chloride in 400 ml. of water. Add 420 ml. of 1:110 hydrochloric acid, mix, and dilute to 1 liter. To a 1-ml. sample of urine containing up to 0.8 mg. of phenylpyruvic acid, add 4 ml. of glycine buffer and 1 ml. of 4% uranyl nitrate solution in glycine buffer. Cool in ice for 10 minutes and centrifuge for 5 minutes to remove phosphates. Cool a 5-ml. portion for 10 minutes in ice. Add 1 ml. of ice-cooled 9.8% ferrous ammonium sulfate hexahydrate solution in glycine buffer and 0.1 ml. of cooled 10% ferric chloride solution. Mix, let stand in ice in the dark for 30 minutes, and read against a reagent blank at 630 mμ.

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[52] M. Polonowski, P. Desgrez, and F. Delbarre, *Bull. soc. chim. biol.* 29, 1049-54 (1947).

[53] K. Kropp and K. Lang, *Klin. Wochschr.* 33, 482-5 (1955).

[54] T. P. The, P. Fleury, and C. L. Vink, *Clin. Chim. Acta* 2, 424-8 (1957); J. P. Berry and L. I. Woolf, *Nature* 169, 202-3 (1952).

[55] A. Saifer and A. F. Harris, *Clin. Chem.* 5, 203-17 (1959).

ASCORBIC ACID, VITAMIN C, AND DEHYDROASCORBIC ACID

Total ascorbic acid and dehydroascorbic acid are determined in trichloroacetic acid solution as dehydroascorbic acid after treatment with Norite and addition of 2,4-dinitrophenyl hydrazine.[56] The Norite serves as an oxidizing agent. Therefore by omission of the oxidizing agent the difference between the two determinations is ascorbic acid.

The same function of Norite as an oxidizing agent can be supplied by 2,6-dichlorophenolindophenol (Vol. III, pp. 366-7) for more rapid determination of ascorbic and dehydroascorbic acid.[57] Thiourea is then added to prevent oxidation of the vitamin by ferric ion or peroxides. But if thiourea is added before the sample contacts the reagent ascorbic acid is not oxidized and dehydroascorbic acid is determined by difference.

The red color can be developed in glacial acetic acid or sulfuric acid. Use of a 2:3 mixture of 85% phosphoric acid and concentrated hydrochloric acid provides a twofold increase in sensitivity over the glacial acetic acid method. In the presence of the phosphoric acid-hydrochloric acid mixture, up to 1.5 mg. of glucose or fructose and up to 0.05 mg. of glucuronolactone do not interfere. At a glucuronolactone concentration of 1.5 mg. turbidity occurs. However, the ratio of glucuronolactone to ascorbic acid must be greater than 10:1 for interference. Sulfuric acid eliminates colors formed by glucose, fructose, or arabinose. 2,3-Diketogulonic acid does not interfere.

To determine ascorbic acid in the presence of ascorbone and diketogulonic acid oxidize with bromine and react with thiourea in metaphosphoric acid.[58] Ascorbone is reduced with potassium citrate and hydrogen sulfide. Thereafter ascorbic acid, ascorbone, and diketogulonic acid are read in different fractions by 2,4-dinitrophenylhydrazine.

In the determination of ascorbic acid with aniline and its derivatives, the presence of a nitro radical in the ortho or para position of the aniline is essential for the production of an intensely colored substance. The methoxy group in the ortho or para position would serve as auxochrome group while hydroxy and amine groups would not.[59] Thus the color developed by ascorbic acid with *p*-nitroaniline is darker than with 4-

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[56] R. R. Schaffert and G. R. Kingsley, *J. Biol. Chem.* **212,** 59-68 (1955); Cf. Chi-Lan Fang *et al., Ying Yang Hsueh Pao* **2,** 97-104 (1957); A. Inczédy, *Elelmezési Ipar* **9,** 177-9 (1955).

[57] M. A. Schwartz and J. N. Williams, Jr., *Proc. Soc. Exptl. Biol. Med.* **88,** 136-8 (1955); V. Ciupersescu, *Rev. ind. aliment., Produse vegetale* **1958,** No. 11, 1-3.

[58] G. H. Sloane-Stanley, *Biochem. J.* **65,** 11P (1957).

[59] H. Fukuba, A. Yamasaki, and C. Inagaki, *Vitamins (Japan)* **9,** 312-6 (1955).

methoxy-2-nitroaniline. Also glutathione, cysteine, glucose, xylose, and tannin interfere with the latter reagent.

Ascorbic acid reduces diazotized 4-methoxy-2-nitroaniline in acid media to produce a yellow product which turns blue in alkaline media.[60] The reaction is carried out in *n*-butanol in preference to ethanol or isopropanol since *n*-butanol is miscible with the aqueous sample solutions in the ratios used. The color is extracted with the *n*-butanol by ethyl ether.[61] Flavonoids present in eightfold excess alter the color to red-brown.[62] Pectic substances when precipitated by ethanol interfere by sorption of some ascorbic acid.

Oxalic acid is preferred over metaphosphoric acid as an ascorbic acid stabilizer in this method because of its greater solubility in the final alcohol solution. The reaction is highly specific for vitamin C in the presence of other vitamins. A straight line curve is obtained for concentrations of 0.4-2 mg. per 200 ml. of final solution but it does not pass through the point of origin. Larger amounts of reagent cause a decrease in color.

The following ratios of reducing agents may be present in relation to 1 part of ascorbic acid: 3 parts of sulfur dioxide, 2 parts of ferrous ion, and 3 parts of stannous ion. Vitamins, hydroascorbic acid, and 2,3-diketogulonic acid do not interfere. Pyruvic acid produces a red color. Twice as much phenol as ascorbic acid can be tolerated. Polyhydroxy alcohols and Tweens do not interfere. Reductines and reductic acid causes high results and may be eliminated by a formaldehyde modification.

By this method, only ascorbic acid is determined. To determine total ascorbic acid including dehydroascorbic acid, a reduction by hydrogen sulfide is required.

Milk, powdered milk, fortified animal feed, urine, blood serum, pharmaceutical preparations, natural juices, and processed foods are analyzed with this reagent. Canned apples, peaches, and figs require a formaldehyde modification for reductones and soluble coffee requires a prior extraction with 0.05% oxalic acid solution in ethanol before analysis. When diazotized *p*-nitroaniline is used the color is read at 540 mμ.[63]

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[60] M. Schmall, C. W. Pifer, and E. G. Wollish, *Anal. Chem.* 25, 1486-90 (1953); Y. Kochi and S. Kasahara, *J. Pharm. Soc. Japan* 74, 443-6 (1954); J. Frágner and J. Davidek, *Průmysl potravin* 7, 76-9 (1957).

[61] M. Schmall, C. W. Pifer, E. G. Wollish, R. Duschinsky, and H. Gainer, *Anal. Chem.* 26, 1521-2 (1954).

[62] J. Davidek and E. Davidková, *Nahrung* 3, 515-9 (1959).

[63] H. Moor, *Mitt. Lebensm. Hyg.* 47, 20-7 (1956).

The determination of excess 2,6-dichlorophenolindophenol (Vol. III, pp. 366-7) after reduction by ascorbic acid has varied modifications. *p*-Chloromercuribenzoic acid acts as a complexing agent for hydrogen sulfide, cysteine, and related interfering sulfhydryl compounds.[64] This reagent has the advantage over mercuric acetate, also used for this purpose, of the elimination of the steps of removal of excess mercuric ions and conversion of the dehydroascorbic acid to ascorbic acid. The pH of the solution must remain above 3.5, since there is a spontaneous fading below this point.[65] Solvents for extraction are amyl acetate,[66] xylene, ethyl acetate, butyl acetate,[67] or butanol.[68]

As little as 0.1 mg. of ascorbic acid per liter can be determined with *p*-sulfophenylhydrazine.[69] Dehydroascorbic acid and diketogulonic acids are also measured at the same time. Ascorbic acid quantitatively reduces picric acid to form picramic acid.[70] The picramic acid so developed can be compared to picramic acid standards.

Ascorbic acid reacts with 3,4-dinitrobenzoic acid to produce a yellow color on acidification with hydrochloric acid. The color is extracted with iso-amyl alcohol.[71]

A method for ascorbic acid depends on the reduction of ferric chloride at pH 1-2 in the presence of orthophosphoric acid which inhibits reductone and other reducing agents. Ferrous ion so formed is subsequently estimated by α,α'-dipyridyl (Cf. Vol. III, p. 367).[72] The test is sensitive to 0.005 mg. of ascorbic acid per ml. and has been applied to orange juice, honey, and urine. Reductone, glucosone, reductic acid, α-tocopherol, glutathione, cysteine, acetol, methyl glyoxal, and creatine do not interfere.

Thionine is a reagent for both ascorbic acid and dehydroascorbic acid. The reaction takes place in sunlight or artificial light.[73] Ascorbic acid serves as the reducing agent to convert phosphomolybdate to molybdenum blue.[74] This is not strikingly different from the corresponding silicomolybdate reagent (Vol. III, p. 368) but the peak absorption is a little higher, at 825-837 mμ. Cloudiness of filtrates in general in the determination of plasma ascorbic acid can be eliminated by use of exactly 0.52 *N* metaphosphoric acid as a protein precipitant.[75] For ascorbic acid by *N*-ethylcarbazole see page 168.

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[64] J. A. Owen, B. Iggo, and D. B. Horn, *Nature* **174**, 701 (1954).

[65] J. A. Owen and B. Iggo, *Biochem. J.* **62**, 675-80 (1956).

[66] H. Imai and T. Fujitani, *Nippon Kagaku Zasshi* **76**, 1213-5 (1955).

[67] M. Arai and T. Kitajima, *Vitamins (Japan)* **9**, 114-7 (1955).

[68] H. Mitsuta and T. Shikanai, *Ibid.* **13**, 394-401 (1957).

[69] J. Baraud, *Bull. soc. chim. France* **1951**, 837-43; **1953**, 521-5.

[70] Gulio Di Bacco, *Boll. chim. farm.* **92**, 115-9 (1953).

[71] T. Takemoto, K. Daigo, and T. Takai, *J. Pharm. Soc. Japan* **75**, 1025 (1955).

[72] M. X. Sullivan and H. C. N. Clarke, *J. Assoc. Offic. Agr. Chemists* **38**, 514-8 (1955).

[73] R. Charonnat and L. Bezanger-Beauquesne, *Ann. pharm. franç.* **16**, 96-102 (1958).

[74] M. L. Tsap, *Biolshimaya* **21**, 534-7 (1956).

Procedure—*Total ascorbic acids by 2,4-dinitrophenylhydrazine. Carbon as oxidizing agent. Whole blood and serum.*[76] Add a 5-ml. sample dropwise to 15 ml. of 6% trichloroacetic acid, mixing continuously. After 5 minutes centrifuge for 10 minutes.

Let a 2% solution of 2,4-dinitrophenylhydrazine in 1:3 sulfuric acid stand overnight and filter. Add 0.5 teaspoonful of Norite to an aliquot of the deproteinized sample to oxidize ascorbic acid to dehydroascorbic acid. Shake vigorously for 1 minute and filter. To 4 ml. of the filtrate containing up to 0.06 mg. of ascorbic acid, add 1 drop of 10% thiourea in 1:1 alcohol to prevent any oxidation from ferric ion or hydrogen peroxide.

Add 1 ml. of 2,4-dinitrophenylhydrazine reagent and heat at 100° for 5 minutes. Cool rapidly and add 5 ml. of 9:1 sulfuric acid dropwise to destroy any colors formed from glucose, fructose, or arabinose. Swirl, let stand for 10 minutes, and read at 505 mμ against a reagent blank containing no dinitrophenylhydrazine solution.

Urine. Add a 1-ml. sample to 19 ml. of 4% trichloroacetic acid solution. Determine as described for whole blood and serum starting at "Let a 2% solution of. . . ." Heat at 100° for 10 minutes instead of 5 minutes.

Vegetables and fruits. Blend a 2-gram sample with 98 ml. of 0.5% oxalic acid for 5-10 minutes. Develop a 20-ml. aliquot starting at "Let a 2% solution of . . . ."

l-Ascorbic acid. Run parallel determinations following the procedure for total ascorbic acid by 2,4-dinitrophenylhydrazine, omitting the Norite in one determination. The difference between the two values obtained is the *l*-ascorbic acid.

Total ascorbic acids and dehydroascorbic acid. 2,6-Dichlorophenolindophenol as the oxidizing agent. Take a 2-ml. sample containing up to 0.03 mg. of ascorbic acid and dehydroascorbic acid. To deproteinize, dilute with 5% metaphosphoric acid solution in 1:9 acetic acid so that the ascorbic acid concentration is 0.005-0.01 mg. per ml. Centrifuge.

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[75] Roderick P. MacDonald, *Tech. Bull. Registry Med. Technologists* **25**, 51 (1955); *Am. J. Clin. Pathol.* **25**, 343 (1955).

[76] R. R. Schaffert and G. R. Kingsley, *J. Biol. Chem.* **212**, 59-68 (1955).

Prepare three tubes as follows: tube 1 for dehydroascorbic acid, tube 2 for total ascorbic acid, and tube 3 as a sample blank. Add 1 drop of 0.04% indophenol reagent and 2 ml. of 1% thiourea in 5% metaphosphoric acid solution to tube 1. Add 2 ml. of sample to tubes 1, 2, and 3. Add 1 ml. of 2% 2,4-dinitrophenylhydrazine in 1:3 sulfuric acid to tubes 2 and 3. Add 2 ml. of 1% thiourea in 5% metaphosphoric acid solution to tubes 2 and 3. Add 1 ml. of 2% 2,4-dinitrophenylhydrazine in 1:3 sulfuric acid to tubes 1 and 2. Incubate all tubes at 37° for 3 hours. Add 5 ml. of a 3:2 mixture of concentrated hydrochloric acid—85% phosphoric acid to all tubes and 1 ml. of 2% 2,4-dinitrophenylhydrazine reagent in 1:3 sulfuric acid to tube 3. Mix and read all at 540 mμ.

Ascorbic acid, diketogulonic acid, and ascorbone by bromine, thiourea, and 2,4-dinitrophenylhydrazine. Brain or cerebrospinal fluid. Dilute 1 gram of ground brain sample with 15 ml. of water or take a 16-ml. sample of cerebrospinal fluid. Add 4 ml. of 2.5% stannous chloride in 5% metaphosphoric acid solution. Centrifuge and mix one-third of the extract with 0.25 volume of 0.5% thiourea in 1% metaphosphoric acid solution to stabilize the ascorbic acid.

For the reduction of ascorbone, mix a second fraction of the extract with 0.1 ml. of 1% potassium citrate solution and treat with hydrogen sulfide for 2.5 minutes. Stopper and heat at 37° for 2.25 minutes. Cool in ice, add 3 drops of concentrated hydrochloric acid, and centrifuge. Mix the supernatant liquid with 0.25 volume of 5% thiourea in 1% metaphosphoric acid solution.

Add bromine water to the remaining third of the extract until yellow to oxidize the ascorbic acid. Bubble nitrogen through until the extract is free from bromine. Mix with 0.25 volume of 5% thiourea solution in 1% metaphosphoric acid solution.

Mix 2-ml. samples of each fraction with 0.5 ml. each of 2% 2,4-dinitrophenyllhydrazine solution in 1:3 sulfuric acid. Incubate at 30° for 15 hours with a blank tube containing 1:3 sulfuric acid. Add 85% sulfuric acid to 5 ml. and read. Fraction 1 contains ascorbone and diketogulonic acid; fraction 2, diketogulonic acid; fraction 3, all three components.

By 4-methoxy-2-nitroaniline.[77] As a reagent, recrystallize 4-methoxy-2-nitroaniline from 95% ethanol and dry at 60°. Dissolve 0.5 mg. of the pure compound in 125 ml. of glacial acetic acid and dilute to 250 ml. with 1:9 sulfuric acid. The reagent is stable for at least 2 months.

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[77] M. Schmall, C. W. Pifer, E. G. Wollish, R. Duschinsky, and H. Gainer, *Anal. Chem.* **26**, 1521-2 (1954).

Add 2 ml. of 0.2% sodium nitrite solution to 2 ml. of reagent. Swirl until the orange color of the amino reagent disappears and add 75 ml. of *n*-butanol. Dissolve a solid sample containing 0.5-2 mg. of ascorbic acid in 5 ml. of 0.5% oxalic acid solution or adjust a sample solution to that acidity. Filter if necessary and add 25 ml. of 10% sodium hydroxide solution and 150 ml. of ether. Shake, allow the layers to separate, and remove the bottom layer. Wash the remaining ether three times with 15-ml. portions of 10% sodium hydroxide solution and dilute the washings and extract to 200 ml. with water. Read at 570 mμ against a reagent blank for colorless samples, and against water for colored samples. Read a sample blank containing sample and all reagents except 4-methoxy-2-nitroaniline. For colored samples subtract the sum of the reagent blank and sample blank from the reading for a corrected value.

By 2,6-dichloroindophenol.[78] *Foodstuffs.* Finely chop a 10-30 gram sample. Blend a 20-25 gram portion with 25 ml. of 2% oxalic acid solution and 5 grams of sea sand. Dilute to 100 ml. with 1% oxalic acid solution. Mix, and centrifuge.

To a 10-ml. aliquot of the centrifugate, add 0.2 ml. of 0.04% bromocresol green solution. Add 4% sodium hydroxide solution dropwise to the green-blue color of the indicator. Add 1 ml. of a phosphate-citrate buffer for pH 4 and 4 ml. of 0.01% solution of 2,6-dichloroindophenol. Shake and let stand for 5 minutes. Extract by shaking with 10 ml. of toluene for 10 seconds. After 5 minutes drain the aqueous layer and dry the pink organic layer with 3 grams of anhydrous sodium sulfate. Stir, let stand for 5 minutes, and filter. Read at 480 mμ.

Sulfhydryl compounds present.[79] To 3 ml. of 1.8% metaphosphoric acid containing up to 0.06 mg. of ascorbic acid add 1 ml. of 0.2% *p*-chloromercuribenzoic acid solution in 0.2% sodium hydroxide solution. Let stand for 5 minutes and centrifuge to remove precipitated excess *p*-chloromercuribenzoic acid. To 2 ml. of the supernatant liquid, add 0.5 ml. of sodium citrate solution of such a concentration as to bring the final pH of the solution to 3.5. Add 1 ml. of 0.004% 2,6-dichlorophenolindophenol solution and read after 30 seconds at 520 mμ.

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[78] V. Ciupersescu, *Rev. ind. aliment., Produse vegetale* **1958**, No. 11, 1-3.

[79] J. A. Owen, B. Iggo, and D. B. Horn, *Nature* **174**, 701 (1954).

By p-*sulfophenylhydrazine.*[80] Titrate a sample containing not more than 10 mg. of ascorbic acid with iodine solution. Add 50 ml. of 2.5% boric acid solution and adjust the pH to 7.4. Dilute to 200 ml. with water and let stand for 2 hours at room temperature to destroy desoxytartaric acid. Take two 10-ml. aliquots. To one add 2 ml. of 0.5% *p*-sulfophenylhydrazine solution and determine the amount of 1:100 hydrochloric acid required to yield a pH of 7.4. After 2 hours, to the other aliquot add 2 ml. of *p*-sulfophenylhydrazine solution and the determined quantity of 1:100 hydrochloric acid for pH 7.4. Heat at 100° for 75 minutes and cool. Dilute to 20 ml. with water and read at 436 mμ. The color due to ascorbic acid will have decomposed in the first tube and the value depends on the difference in reading between the two tubes.

By picric acid.[81] Dissolve 0.235 gram of picramic acid in 400 ml. of water and add 25 ml. of 2% sodium hydroxide solution. Heat and dilute the cooled solution to 1 liter. Mix a 5-ml. sample containing up to 5 mg. of ascorbic acid with 3 ml. of 0.1% picric acid solution and 2 ml. of 2% sodium hydroxide solution. Mix 8 ml. of the picramic acid solution with 1.8 ml. of 2% sodium hydroxide solution and 0.2 ml. of water. Heat both mixtures at 100° for 3 minutes, cool, and read. Five mg. of ascorbic acid reduces picric acid to form 1.884 mg. of picramic acid which is the amount in the standard used.

By 3,4-dinitrobenzoic acid.[82] As a reagent, dissolve 2 grams of 3,4-dinitrobenzoic acid and 31.8 grams of sodium carbonate in 1 liter of water. Treat an aliquot containing up to 1 mg. per ml. of ascorbic acid with the reagent for 10 minutes at 15-20°. Acidify the product with hydrochloric acid to produce the yellow color. Extract with isoamyl alcohol and read at 415 mμ. Beer's law is followed.

By α,α′-dipyridyl and ferric ion.[83] To a 1-ml. aliquot containing 0.02 mg. ascorbic acid, add 0.3 ml. of concentrated orthophosphoric acid to bring the pH to 1-2. Add 5 ml. of 0.5% α,α′-dipyridyl solution and 1 ml. of 1% ferric chloride solution. Let stand for 15 minutes and read the transmittance.

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[80] J. Barand, *Bull. soc. chim. France* **1951,** 837-43.

[81] Guilio Di Bacco, *Boll. chim. farm.* **92,** 115-9 (1953).

[82] T. Takemoto, K. Daigo, and T. Takai, *J. Pharm. Soc. Japan* **75,** 1025 (1955).

[83] M. X. Sullivan and H. C. N. Clarke, *J. Assoc. Offic. Agr. Chemists* **38,** 514-8 (1955).

By thionine. Ascorbic acid. Grind a solid sample with an equal amount of ammonium sulfate and extract with a saturated ammonium sulfate solution. Saturate liquid samples with ammonium sulfate and filter. Mix a 5-ml. portion of the sample solution containing at least 0.025 mg. of ascorbic acid with 5 ml. of water and 3 ml. of 0.005% thionine solution. Add 1 ml. of 1:3 sulfuric acid and expose to direct sunlight or to light of a 75-watt bulb 25 cm. away. Read the maximum color.

Dehydroascorbic acid. As a buffer for pH 4 mix 45 ml. of 1:110 hydrochloric acid with 55 ml. of a solution of 21 grams of citric acid and 200 ml. of 4% sodium hydroxide solution and dilute to 1 liter with water. Mix 7.5 ml. of the extract with 2.5 ml. of the pH-4 buffer. Add 0.5 ml. of 10% sodium sulfide solution, mix, and let stand for 1 hour. Add 0.5 ml. of 1:3 sulfuric acid, shake and filter after 10 minutes. Determine the ascorbic acid in the filtrate from "Mix a 5-ml. extract . . ." to give a total of ascorbic and dehydroascorbic acids. Determine dehydroascorbic acid by difference.

3,7,12-TRIHYDROXYCHOLANIC ACID, CHOLIC ACID

Cholic acid and desoxycholic acid are determined spectrophotometrically at 320 mμ and 385 mμ respectively after heating in 2:1 sulfuric acid to conjugate.[84] The absorption conforms to Beer's law. Reading at 318 mμ and 350 mμ is an alternative.[85] However, the optical density at 318 mμ due to desoxycholic acid is not stable while that due to cholic acid is very stable, and it is difficult to determine one acid in the presence of the other. Since chenodesoxycholic acid, found in the bile of man, the rat, and the chicken has an almost identical absorption spectrum to that of desoxycholic acid, the readings at 385 mμ for these samples may be due to this acid or to both acids in the case of human bile samples. All samples are hydrolyzed because of the differences between the absorption spectra of the free acids and their conjugates. Lithocholic acid and α-hyodesoxycholic acid do not interfere.

When cholic acid is heated in phosphoric acid and diluted with butanol it gives a blue fluorescence and absorbs in the ultraviolet.[86] Desoxycholic acid does not so react.

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[84] E. H. Mosbach, H. J. Kalinsky, E. Halpern, and F. E. Kendall, *Arch. Biochem. and Biophys.* **51**, 402-10 (1954).

[85] Lawrence C. Kier, *J. Lab. Clin. Med.* **40**, 755-65 (1952); P. Engelbertz and E. Babel, *Zentr. Arbeitsmed. u. Arbeitsschultz* **3**, 161-8 (1953); P. Metals, H. Deiss, and J. Warter, *Bull. soc. chim. biol.* **39**, 321-9 (1957).

[86] R. Willemart and J. Ferdet, *Ann. pharm. franç.* **17**, 366-7 (1959).

Hydroxamic acid derivatives of bile acids develop a purple color upon addition of ferric perchlorate.[87] Another reagent for bile salts is furfural in acetic acid.[88] Beer's law is followed for bile substances up to 0.4 mg.

Procedure—*Direct reading of cholic acid and desoxycholic acid. Bile.* Heat a 1-ml. or smaller sample containing approximately 1 mg. of total bile acids to boiling with 5 ml. of absolute ethanol. Filter, and rinse with two 5-ml. portions of hot absolute ethanol. Evaporate the ethanol at 100° under a current of air. Hydrolyze in an autoclave at 15-lb. pressure with 5 ml. of 5% sodium hydroxide solution for 3 hours. Cool to room temperature and acidify with concentrated hydrochloric acid using Congo red as an indicator. Extract four times with 10-ml. portions of ether and wash the combined extracts with 2 ml. of water. Dry the ether over sodium sulfate for 2-3 hours. Filter, and wash the sodium sulfate with 5 ml. of ether. Evaporate the ether at 100° and dissolve the residue in 10 ml. of acetone. Evaporate the acetone from a 1-ml. aliquot containing up to 0.1 mg. of bile acids to dryness. Add 5 ml. of 2:1 sulfuric acid and heat for 15 minutes at 60°. Rapidly cool to room temperature and let stand for 15 minutes from the time the heating period ended. Read cholic acid at 320 mμ and desoxycholic acid at 385 mμ within 1 hour. Some improvement in accuracy is attainable by the use of simultaneous equations.

Plasma or serum. Dilute a 0.2-ml. sample with a 1:1:1 mixture of absolute ethanol, acetone, and ether to 5 ml. Heat at 60° for 3 minutes and centrifuge. Wash the precipitate twice with 5-ml. portions of the solvent mixture. Evaporate the supernatant liquid and washings containing up to 0.1 mg. of bile acids to dryness and complete as for bile starting at "Add 5 ml. of 2:1 sulfuric acid. . . ."

By ferric perchlorate. Bile salt. Acidify a sample containing 0.2-10 micromoles of cholic or dihydroxycholanic acid to Congo red with 1:9 hydrochloric acid. Extract three times with 5-ml. portions of ether and wash the combined extracts 5 times with 4-ml. portions of 1:6 hydrochloric acid. Reserve the ether phase. Cool the acid extract and treat with 3 ml. of 60% sodium hydroxide solution. Extract three times with 10-ml. portions of ether and wash the extracts 3 times with 2-ml. portions of water. Filter the ether extract and dry with anhydrous sodium sulfate as the cholic acid sample.

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[87] T. Usui, H. Otagaki, and K. Shimizu, *Yonago Acta Med.* 2, 52-4 (1957).
[88] A. Crocetta, *Accad. med.* (*Turin*) **68**, 128-31 (1953).

Extract the reserved ether phase with 1:3 hydrochloric acid and cool the extract. Treat with 6 ml. of 60% perchloric acid solution and dilute to 100 ml. with 10 ml. of water and the balance methanol. Mix 40 ml. of this solution with 10 ml. of water, 10 ml. of methanol, and 18 ml. of 60% perchloric acid solution. Dilute to 1 liter with methanol.

Treat the cholic acid and dihydroxycholanic acid fractions with an excess of diazomethane in ether. Store overnight and evaporate. Reflux the residue 5 minutes with 1.5 ml. of a freshly prepared and filtered solution of equal volumes of 12.5% hydroxylamine hydrochloride solution in methanol and 12.5% sodium hydroxide solution in methanol. Cool and dilute to 25 ml. with ferric perchlorate reagent. Let stand for 10 minutes and read against the hydroxylamine hydrochloride reagent at 530 mμ. An appropriate calibration curve is constructed from 0.2-10 micromoles of cholyl hydroxamic acid and ferric perchlorate.

By furfural. Serum. Shake a 5-ml. sample with 5 ml. of barium salt solution prepared by mixing 100 ml. of saturated barium hydroxide solution with 0.7 ml. of glacial acetic acid. Let stand for 10 minutes and add 35 ml. of absolute ethanol. Shake and heat at 100° for 3 minutes. Stopper and store for 12 hours at 4°. Dilute to 50 ml. with 95% ethanol at 4° and filter. Evaporate 30 ml. of the filtrate and take up the residue in 3 ml. of glacial acetic acid. Prepare four centrifuge tubes, two containing 1 ml. each of the solution and two containing 1 ml. of 0.1% cholic acid solution in acetic acid. Add 6 ml. of concentrated phosphoric acid to all tubes and 1 ml. of 0.56% furfural solution in 1:1 acetic acid to one sample tube and one cholic acid tube. To the remaining tubes add 1 ml. of 1:1 acetic acid. Shake and heat at 67° for 30 minutes. Cool rapidly and dilute to 10 ml. Read at 610 mμ.

By phosphoric acid. Dissolve a sample expected to contain 100 mg. of cholic acid in 2 ml. of 0.8% sodium hydroxide solution. Add 10 ml. of 85% phosphoric acid and heat at 40° for 90 minutes. Cool, dilute to 25 ml. with butanol, and read at 260 mμ.

ACETYLCHOLINE

Hydroxylamine reacts with acetylcholine to convert it rapidly and quantitatively to a hydroxamic acid.[89]

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[89] Shlomo Hestrin, *J. Biol. Chem.* 180, 249-61 (1949).

Procedure—As reagent mix equal parts of 3.5% sodium hydroxide solution and 6.6% aqueous hydroxylamine. Add 2 ml. of this to 1 ml. of sample. After 1 minute or more adjust the pH to 1.2 ± 0.2 with 1 ml. of 1:2 hydrochloric acid. Add 1 ml. of 10% solution of ferric chloride hexahydrate and read at 540 mμ. As a correction for background color treat a sample first with acid, then with the reagent, then with the ferric chloride. No hydroxamic acid is formed under those conditions.

3,7,12-TRIKETOCHOLANIC ACID, DEHYDROCHOLIC ACID

Both *m*-dinitrobenzene (Vol. III, page 377) and salicylaldehyde are reagents for dehydrocholic acid.[90] Beer's law is followed.

Procedure—*By* m-*dinitrobenzene.* Dilute a sample containing 0.16-0.4 mg. per ml. of dehydrocholic acid to 2 ml. with 95% ethanol. Add 1 ml. of 2% *m*-dinitrobenzene solution in 95% ethanol, and 1 ml. of 2.8% potassium hydroxide solution. Read at 530 mμ.

By salicylaldehyde. Treat a 1-ml. sample containing 3130 mg. of dehydrocholic acid in 95% ethanol with 2 ml. of 50% sodium hydroxide solution. Add. 0.5 ml. of 5% salicylaldehyde solution in 95% ethanol. Heat at 37° for 10 minutes and read at 460 mμ.

CHENODESOXYCHOLIC ACID

A specific reaction for chenodesoxycholic acid involves sulfuric acid in ethyl acetate. The acid is extracted from the sample with ether.[91]

Sample—*Bile.* Hydrolyze a 0.2-1 ml. sample of fresh bile or 25-100 mg. sample of lyophylized bile with 30 ml. of 4% sodium hydroxide solution for 5 hours at 110-20°. Neutralize with 1:5 hydrochloric acid, adding 1 ml. in excess. Dilute to about 50 ml. with water and extract with three 50-ml. portions of ether. Reduce the combined ether extracts to about 50 ml. and wash with water until the water is free from hydrochloric acid as indicated by tests with 0.85% silver nitrate solution in 1:4 nitric acid. Evaporate the ether solution to dryness and extract the residue three times with a 1:1 mixture of 70% ethanol and hexane. Evaporate the combined extracts to dryness, dissolve in absolute ethanol, and dilute to 10 ml.

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[90] C. Bergamini and W. Veronese, *Sperimentale, Sez. chim. biol.* 4, 79-84 (1953).

[91] Bjorn Isaksson, *Acta Chem. Scand.* 8, 889-97 (1954).

Procedure—*Macro.* Evaporate an aliquot containing 0.2-1.5 mg. of chenodesoxycholic acid to dryness at 100° and heat for 5 minutes. Cool to room temperature and add 3 ml. of 15:1 ethyl acetate-concentrated sulfuric acid. Add 2 ml. of acetic anhydride, mix, and read at 615 mμ 12-15 minutes after addition of acetic anhydride.

Micro. Follow the procedure for the macro method using a sample containing 0.01-0.15 mg. of chenodesoxychloric acid, 1 ml. of 15:1 ethyl acetate-sulfuric acid, and 0.67 ml. of acetic anhydride. Read after 12-15 minutes at 615 mμ.

7-Keto-3,12-dihydroxycholanic Acid

Saturated acids, in general, do not absorb ultraviolet light. However, a saturated cholanic acid possessing one or more keto groups can be determined by ultraviolet spectrophotometry. The absorbancy is proportional to the number of carbonyl groups if the groups are separated from each other by three or more methylene units.[92]

Procedure—*Oxidation mixture of cholic acid.* Dilute a sample containing the equivalent of 100 mg. of 7-keto-3,12-dihydroxycholanic acid to 25 ml. with 0.4% sodium hydroxide solution. Read against sodium hydroxide solution at 285 mμ, 275 mμ, and 300 mμ. Calculate as mg. of 7-keto-3,12-dihydroxycholanic acid per ml. as follows:

$$\text{corrected absorbancy} = (4.29 \times A_{285}) - (2.574 \times A_{275}) - 1.716 \times A_{300})$$

$$\frac{\text{mg. keto acid}}{\text{ml.}} = \frac{\text{corrected absorbancy} \times 404.5 \times 25}{35.6 \times \text{volume of sample taken in ml.}}.$$

Gluconic Acid

Gluconic acid is usually found in an alkaline medium. Therefore a simple method for its determination makes use of the color of the copper-gluconate complex in alkaline medium.[93] Heating the solution increases the selectivity of the method as various complexes formed by other compounds with copper are thermally unstable. Heating also helps to coagulate colloidal dispersions and aids filtration of the cupric hydroxide.

Limiting factors include the presence of compounds which will complex with the reagent and sorption of the complex by the cupric hydroxide precipitate. Reducing sugars are the most serious interferences since they both reduce the copper gluconate complex and the products of this oxidation may form stable copper complexes. Ammonium salts and cyanides interfere. They can be removed by acidification with acetic acid and heating to remove hydrogen cyanide. Boiling the alkaline sample removes ammonium salts. Phosphate ions do not interfere. Alkali metal hydroxides need not be removed from the sample provided the sodium hydroxide concentration in the standard is adjusted to account for their presence. Beer's law is followed.

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[92] E. L. Pratt, *Anal. Chem.* **25**, 175-7 (1953).

[93] Leslie L. Alt, *Ibid.* **27**, 749-51 (1955).

Procedure—To a sample containing 0.01-0.1 mg. of gluconic acid add 15 ml. of 0.8% copper sulfate pentahydrate solution. Add 10 ml. of 10% sodium hydroxide solution. Boil for 5 minutes and cool to room temperature. Dilute to 50 ml. and centrifuge a part. Read the centrifugate at 660 mμ against 2% sodium hydroxide solution.

Alternatively, treat a sample containing 0.01-0.1 mg. of gluconic acid with 18 ml. of 5% sodium hydroxide solution. Slowly add 0.8% copper sulfate pentahydrate solution with stirring to redissolve the precipitate formed upon addition of the sodium hydroxide. The presence of a permanent precipitate indicates that the chelating capacity of the gluconate is exhausted. Avoid formation of an unduly large cupric hydroxide precipitate. Boil for 5 minutes to decompose other copper complexes formed and to coagulate the free cupric hydroxide. Filter through an ultrafine filter. Wash with 2 ml. of 5% sodium hydroxide solution and dilute to 50 ml. Read at 660 mμ against 2% sodium hydroxide solution.

FORMAMINO-*l*-GLUTAMIC ACID
N-FORMAMIDOYLGLUTAMIC ACID

This intermediate in the metabolism of histidine is enzymatically converted in the presence of tetrahydrofolic acid to 5,10-methenyltetrahydrofolic acid. The latter is read in the ultraviolet at a wave length selected according to the concentration present.[94]

Procedure—For preparation of the enzyme homogenize 100 grams of frozen hog liver with 500 ml. of acetone for 1 minute. Filter rapidly on a Büchner funnel. Homogenize the moist filter cake with acetone and refilter. Dry the coarsely screened moist cake at room temperature and store *in vacuo* at −20°.

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[94] H. Tabor and L. Wyngarden, *J. Clin. Invest.* 37, 824-8 (1958).

To extract stir 160 grams of the powder with 1600 ml. of water. Centrifuge in an angle centrifuge after 15 minutes and use the supernatant layer at 1-3°. Add 300 grams of ammonium sulfate to 1400 ml. of the extract. Centrifuge after 15 minutes. Suspend the precipitate in 30-50 ml. of water and store overnight. Centrifuge for 2 hours and discard the supernatant layer. Dissolve the precipitate in 38 ml. of 2.72% sodium acetate trihydrate solution. Centrifuge after 4 hours and discard any precipitate. This is the enzyme for use in the procedure. It contains transferase and cyclodeaminase. It can be stored at 0° for 3 months.

As tetrahydrofolic acid reagent dissolve 88 mg. in 18 ml. of 8.6% mercaptoethanol solution and 2 ml. of 1.12% potassium hydroxide solution. Store in the dark at 0°.

To a portion of urine containing less than 0.1 micromole of formaminoglutamic acid add 0.25 ml. of the enzyme solution and 0.1 ml. of the tetrahydrofolic acid reagent. Add 0.1 ml. of molar phosphate buffer for pH 7.2 and dilute with water to 1 ml. Incubate at 25° for 30 minutes in parallel with a control from which the enzyme was omitted. Add 0.3 ml. of 10% perchloric acid. After 120-135 minutes centrifuge and read at 350 or 365 mμ against the control. If the absorption is too great read at 380 mμ. Correct for an enzyme blank.

1-NAPHTHYL-*N*-METHYLCARBAMATE, SEVIN

Alkaline hydrolysis of 1-naphthyl-*N*-methylcarbamate produces 1-naphthol which is extracted from an acid medium with chloroform when reacted with *p*-nitrobenzenediazonium fluoborate in alkaline solution this yields a color with a maximum absorbance at 590 mμ.[95] The reagent is unstable after 3 hours. Plant tissues are extracted with chloroform. Interfering colors, such as appear in the analysis of pears, can be eliminated by using a 2:1 alumina-Celite column with chloroform as the eluent.

Samples—*Apples.* Grind the sample with anhydrous sodium sulfate to produce a dry crumbly mass. Extract for 30 minutes with chloroform and develop with *p*-nitrobenzenediazonium fluoborate.

Leaves. Extract surface deposits by shaking for 5 minutes with chloroform. Develop with *p*-nitrobenzenediazonium fluoborate.

Pear, peach, and grape. Homogenize the sample with chloroform in a blender. Pass the chloroform phase through anhydrous sodium sulfate to dry and develop with *p*-nitrobenzenediazonium fluoborate.

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[95] R. Miskus, H. T. Gordon, and D. A. George, *J. Agr. Food Chem.* 7, 613-4 (1959).

Procedure—*By* p-*nitrobenzenediazonium fluoborate.* Concentrate a chloroform extract of the sample containing 0.005-0.04 mg. of 1-naphthyl-*N*-methylcarbamate to about 3 ml. and continue to evaporate just to dryness with an air stream. Avoid continued blowing of air after the solvent has evaporated to avoid loss of 1-naphthyl-*N*-methylcarbamate. Take up the residue in 5 ml. of anhydrous methanol with gentle warming. Add 5 ml. of 2% ammonium chloride solution and cool in a freezer for 5 minutes. Filter to remove fruit waxes and rinse twice with a total of 5 ml. of a 1:1 mixture of methanol and water which has been cooled to 5°. To hydrolyze the 1-naphthyl-*N*-methylcarbamate to 1-naphthol add 25 ml. of 2% sodium hydroxide solution and shake. Acidify after 3 minutes with 10 ml. of 19.6% phosphoric acid solution. Mix and extract the 1-naphthol with 25 ml. of chloroform for 1 minute. Concentrate the extract to about 3 ml. and evaporate just to dryness with an air stream, avoiding overblowing of air after solvent evaporation. Take up in 5 ml. of methanol with gentle warming. Add 1 ml. of 2% sodium hydroxide solution and 1 ml. of 0.01% *p*-nitrobenzenediazonium fluoborate solution in methanol. Dilute to 10 ml. with methanol, filter, and read after 30 minutes at 590 mμ against methanol.

CHAPTER 15

POLYBASIC ALIPHATIC ACIDS AND THEIR ESTERS

Mesoxalic acid reacts with 0.05% 2,4-dinitrophenylhydrazine solution in 1:10 hydrochloric acid at 60° for 40 minutes to form its 2,4-dinitrophenylhydrazone.[1] Addition of 10% sodium hydroxide solution converts the hydrazone to a red quinoidal ion with a maximum at 460 mμ. Beer's law is followed.

The reaction of mesoxalic acid with 2,4-dinitrophenylhydrazine is applicable to determination of dihydroxymalonic acid ethyl ester. React the ester in 1:2 hydrochloric acid with 0.2% 2,4-dinitrophenylhydrazine at 60° for 1 hour. Since isopropyl-N-phenylcarbamate is determined by hydrolysis to aniline it appears in Vol. IVA, Chapter 5.

Dicarboxylic Acids and Derivatives

Carboxylic acids react with aqueous hydroxylamine hydrochloride to form hydroxamic acids which are estimated by their color with ferric ion.[2] (See page 289.) The structure of the acid or its derivative affects the speed of the reaction. Dicarboxylic acid amides and esters whose carboxyl groups are separated by one or two carbon atoms, except esters of fumaric acid, give reaction rates which are not always in direct relation to that of their parent acid compound, or in relation to comparable monocarboxylic acid derivatives. Phthalamide, malonamide, succinamide, and maleamide yield roughly half the color of comparable monocarboxylic acid derivatives and their speed of reaction is as follows: acetamide and succinamic acid require 6.5 hours for maximum hydroxamic acid formation, phthalamide requires 2 minutes and maleamide, malonamide and succinamide require 1-2.3 hours. Diethyl maleate has approximately half the color yield found for an equimolar solution of *trans* diethylfumarate. Diethyl malonate, dimethyl succinate, and dimethyl *o*-phthalate have considerable less than twice the absorbances found for comparable equimolar monocarboxylic acid esters. Of the cyclic imides, phthalimide, phthalylglycine, phthalyl-*dl*-methionine, phthalyl-*dl*-phenylalanine, and succinimide yield approximately one equivalent of hydroxamic acid, but the reaction time for the first 4 compounds is 2 minutes while the reaction time for the last compound is 2 hours. Diethyl fumarate and dicarboxylic acid amides and esters that have more than two carbon atoms between the carboxyl groups react with normal speeds and produce more than 1 equivalent of hydroxamic acid.

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[1] S. Mizukami and T. Amano, *Shionogi Kenkyusho Nempo* 7, 533-8 (1957).

[2] V. Goldenberg and P. E. Spoerri, *Anal. Chem.* 31, 1735-7 (1959).

Procedure—See page 289.

OXALIC ACID

In acetic acid medium, the ferric ion in the presence of salicylate yields complexes whose constitution depends on the pH of the medium. When the oxalate ion increases the ferric ion decreases as the complex $OxFe^+$ is formed from the complex RFe^+. Therefore, oxalate may be determined indirectly by the decrease in optical density of the ferric-salicylate complex in the presence of oxalate.[3] The method is reliable in the concentration range of 0.0025-0.025 mg. of oxalate per ml. The sample solution may be neutral, weakly acidic with acetic acid, or slightly alkaline with ammonium hydroxide. If the acidity is not due to acetic acid the solution must be neutralized. An excessive increase in the hydrogen-ion concentration shifts the equilibrium of the system, giving a partial destruction of the ferric-salicylate complex. Tartrate does not interfere even when present in concentrations greater than that of oxalate. Citrate interferes. Beer's law is followed.

A pink or red compound is formed by the reaction of oxalic acid and indole which conforms to Beer's law in a concentration range of 0.05-1 mg. per ml.[4] Formic acid produces a pink or red compound with the reagent. Acetic, propionic, tartaric, citric, benzoic, and uric acids in amounts of 10 mg. per ml. and monobasic potassium phosphate in amounts of 0.4-8 mg. per ml. cause no interference. A ratio up to 6.6 mg. of sodium chloride to 1 mg. of oxalic acid produces no interference. The color of the complex is stable for 24 hours. Sulfuric acid above 1:10 results in less color development and reduced sensitivity. The microdetermination of oxalic acid by an activated reaction requires 5-diphenyl carbazide as a developing agent.[5]

Procedure—*Indirectly by ferric-salicylate.* To prepare the reagent, mix 500 ml. of an iron standard solution containing 0.1 mg. of ferric oxide per ml. (Vol. II, p. 306) with 250 ml. of 1% sodium salicylate. Add 1:1 ammonium hydroxide dropwise until the yellow color appears, adding 10 drops in excess. Dilute to 1 liter with 1:1 acetic acid. Add 20 ml. of the reagent containing 1 mg. of ferric oxide to a 20-25 ml. aliquot of sample containing 0.0025-0.025 mg. of oxalate. Read at 530 mμ and calculate from the reduction in color intensity.

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[3] F. Burriel-Marti, J. Ramirez-Munoz, and E. Fernandez-Caldas, *Ibid.* **25**, 583-5 (1953).

[4] J. Bergerman and J. S. Elliot, *Ibid.* **27**, 1014-5 (1955).

[5] G. Almassy and I. Dezso, *Magyar Kem. Folyoirat* **61**, 107-9 (1955).

By indole. Dissolve the sample in 1:35 sulfuric acid. Take a 2-ml. aliquot containing 0.2-2 mg. of oxalic acid. Run 2 ml. of 0.1% indole solution in concentrated sulfuric acid down the side of the tube to minimize heat development. After 1 minute, mix thoroughly. Heat at 80-90° for 45 minutes, cool, and read at 525 mμ against a reagent blank.

By s-*diphenylcarbazide.* As a reagent, dissolve by warming 10 grams of phthalic anhydride in 175 ml. of redistilled 95% ethanol. Cool, add a solution of 0.625 gram of *s*-diphenylcarbazide in 50 ml. of redistilled 95% ethanol, and dilute to 250 ml. with ethanol.[6]

To a 10-ml. sample add 0.5 ml. of 85% phosphoric acid, 1 ml. of 10% sodium carbonate solution, and 1 ml. of manganese solution containing 1 gram of manganese per ml. (Vol. II, p. 389). Add 0.5 ml. of 0.02% potassium dichromate solution, shake, and heat for 10 minutes at 100°. Add 2 ml. of diphenylcarbazide reagent and dilute to 50 ml. Let stand for 5 minutes and read at 530 mμ against water.

OXALOACETATE

Determination of small quantities of oxaloacetate in the range of 0.0004-0.004 mg. per ml. is possible in concentrations of acetoacetate of 0.1 mg. per ml. by coupling with diazotized *p*-nitroaniline at pH 4.9-5. Both oxaloacetate and acetoacetate react with the diazo reagent to form compounds with almost identical spectra, but the oxaloacetate compounds are unstable.[7] The addition of acid to the reaction mixture effects complete extraction of the resulting color into ethyl acetate. A delay in the extraction over 7.5 seconds may result in loss of as much as 20% of the color. Once extracted, the color is stable for 6 hours.

Addition of sodium nitrite at acid pH removes ascorbic acid interference. The following compounds in concentrations of 5 micromoles do not develop color with the reagent: glycine, alanine, β-alanine, leucine, serine, threonine, cysteine, methionine, aspartic acid, asparagine, glutamic acid, lysine, arginine, histidine, phenylalanine, proline, hydroxyproline, glutathione, citrulline, glucose in concentrations of 50 micromoles, sucrose in concentrations of 50 micromoles, glucose-1-phosphate, fructose, sodium gluconate, carbamyl phosphate, 3-phosphoglyceric acid, α-glycerophosphate, glyoxal, oxalic acid, lactic acid, pyruvic acid, β-hydroxybutyric acid, citric acid, succinic acid, fumaric acid, malic acid, levulinic acid, sodium oleate, sodium crotonate, diphosphopyridine nucleotide, adenosine triphosphate, guanine, xanthine, thymine, uracil, acetoin, acetone, ethanol, urea, glycocyamine, calcium pantothenate, inositol, folic acid, vitamin B_{12}, calciferol, α-tocopherol, choline, glycerol, and cholesterol. Vitamin B_6 group compounds would interfere with the reagent under normal conditions. However, these compounds are normally present in tissue extracts tightly bound to protein and therefore do not interfere in this method.

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[6] B. Saltzman, *Anal. Chem.* **24**, 1016-20 (1952).

[7] P. G. Walker, *Biochem. J.* **58**, 699-703 (1954); G. Kalnitsky and D. F. Tapley, *Ibid.* **70**, 28-33 (1958).

The following compounds react under the conditions of the technic with the reagent: ribonucleic acid, desoxyribonucleic acid, glycogen, vitamin A acetate, ascorbic acid, riboflavin, thiamine, tyrosine, tryptophan, ethanolamine, creatinine, diacetyl, acetoacetate, *p*-hydroxyphenylpyruvate, α-oxoglutarate, and dihydroxymaleic acid. However, they are not decolorized in acid and therefore do not interfere.

Procedure—*Liver tissue.* Remove the liver rapidly from the animal and plunge into liquid oxygen. Weigh the frozen liver, crush at −10°, and homogenize with 4 volumes of cold 2.75% trichloroacetic acid solution at 0-5°. Centrifuge for 5 minutes and develop a portion of the supernatant liquid.

To prepare the reagent, dissolve 0.05 gram of *p*-nitroaniline in boiling water and add the required amount of hydrochloric acid so that the solution is in 1:240 hydrochloric acid. Add 3 ml. of 0.5% sodium nitrite solution to 20 ml. of cold *p*-nitroaniline solution. Keep in an ice bath and add 7 ml. of 1.6% sodium acetate solution just before use. To two 2-ml. sample solutions, add 0.4 ml. of 0.25% sodium nitrite solution to remove ascorbic acid. After 30 seconds add 2 ml. of a solution containing 16.4% of sodium acetate and 12% of acetic acid to buffer pH 5.2 and immediately add 3 ml. of cold diazo reagent. Incubate for 20 minutes at 25° and cool in an ice bath for 3 minutes. To the first tube, add 6 ml. of ethyl acetate and 6 ml. of 1:1 hydrochloric acid to determine the color formed by the reagent and both oxaloacetate and acetoacetate. Within 7.5 seconds extract the color into this ethyl acetate by shaking 30-35 times. In the second tube, destroy the color produced by oxaloacetate and the reagent by adding 6 ml. of 1:1 hydrochloric acid and cooling in ice for 8 minutes. Extract the remaining color with 6 ml. of ethyl acetate.

Carry a reagent blank containing 2 ml. of 3% trichloroacetic acid solution in place of the sample, through the above procedures. Heat 2 ml. of sample extract at 100° for 5 minutes to destroy both oxaloacetate and acetoacetate. Cool rapidly and treat in the same way as for the second tube. Read each tube at 450 mμ against the appropriate reagent blank. The difference between the reading of tubes 1 and 2 represents the color produced by reaction of the reagent with oxaloacetate. The difference in reading between tubes 2 and 3 represent the color produced by acetoacetate.

Succinic Acid

The reaction of succinic acid with sulforesorcinol produces a rose color accompanied by a very strong green fluorescence.[8] Acetic acid, oxalic acid, aspartic acid, and lactic acid do not react. Glutamic acid and tartaric acid give a red color but no fluorescence is observed after addition of ammonium hydroxide. Since glycine, alanine, arginine, cysteine, histidine, and leucine give no color, the procedure can be adapted to protein hydrolyzates.

Procedure—*By resorcinol.* Heat 1-10 mg. of succinic acid, 10 mg. of colorless resorcinol, and 1 ml. of concentrated sulfuric acid at 170° in an oil bath for 6 minutes. Cool, add 13 ml. of concentrated ammonium hydroxide dropwise, and read the rose color after the reaction subsides.

By hydroquinone. Follow the procedure for phthalic acid (page 404), using a sample containing 4-25 mg. of succinic acid. Heat at 135° and extract the red color with 40 ml. of benzene so that the final dilution is 50 ml. Read at 520 mμ.

Tartronate, Hydroxymalonate

The absorbance of glycolic acid, formed by the decarboxylation of tartronate by sulfuric acid, is read after reaction with 2,7-dihydroxynaphthalene.[9] Corrections are applied for the potassium carbonate added, any decarboxylated tartronic acid in the standard solution, and lead tartronate.

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[8] R. Babin, *Bull. soc. pharm. Bordeaux* 92, 173-4 (1953).

[9] Laurence G. Wesson, *Anal. Chem.* 30, 1080-3 (1958).

Sample—*Animal and plant tissue.* Extract a 1-10 gram ground or thinly sliced sample with one or more portions of acetone. This is part of the sample. Expel acetone remaining in the sample *in vacuo,* moisten with water, and freeze. Melt and homogenize in an ice-water bath. Dialyze into 1 liter of water in a refrigerator for 3 days and into a second liter of water for 2 additional days to test the completeness of the dialysis. These dialyzates are the balance of the sample.

Combine the acetone extracts, filter, and evaporate the acetone at room temperature. Hydrolyze the lipides in the residue by stirring with an excess of a thin slurry of 0.15 gram of calcium hydroxide per gram of the oil. The amount required must be estimated from the nature of the sample. Heat at 100° for 15 minutes and let stand overnight. Centrifuge off the insoluble soaps and unsaponifiable matter. Wash well with water and filter the combined supernatant liquids. Add the filtrate containing calcium tartronate equivalent to up to 0.1 mg. of tartronic acid per ml. of saturated calcium hydroxide solution at 25° to the dialyzates. Adjust the pH to at least 8 with 21% potassium carbonate solution, and evaporate to dryness.

Take up the residue with sufficient dilute acetic acid to give a pH of 4.5. Filter and adjust the pH to 5.5 with 12% sodium hydroxide solution. Precipitate by adding a saturated lead acetate solution in 1:4 acetic acid dropwise until precipitation is complete as shown by repeated testing and centrifuging. Measure the volume of the supernatant liquid at this point to permit later calculation of the weight of tartronic acid in solution. To eliminate the lead, stir the precipitate intermittently with 1 ml. of 1:11 sulfuric acid for 30 minutes. Centrifuge and wash the lead sulfate several times.

Adjust the pH of the combined supernatant layers to 5 with 12% sodium hydroxide solution and filter off the small amount of lead sulfide formed. Free the solution from hydrogen sulfide by bubbling filtered air through it. Make alkaline to litmus paper and dilute to 5 ml. or 10 ml. Filter and evaporate a 0.1-0.3 ml. aliquot to dryness at 100° using a stream of filtered air. Similarly evaporate a portion of standard tartronate solution containing 0.01-0.03 mg. of tartronate.

Add 1 ml. of 1:11 sulfuric acid to each tube and expel the carbon dioxide by a current of filtered air. Seal both tubes and heat for 15-16 hours at 100°. Cool and transfer to hard glass tubes with 1 ml. of 21% potassium carbonate solution and wash water. Evaporate to dryness These tubes are AD (acid and dialyzate) and AS (acid and standard) To a third tube, add dialyzate as for the sample and to a fourth add standard tartronate as for the standard. Evaporate both to dryness. These are D (dialyzate) and S (standard).

To prepare the reagent, dissolve 20 mg. of 2,7-dihydroxynaphthalene in 100 ml. of concentrated sulfuric acid and allow the greenish yellow solution to decolorize itself at room temperature. Add 5 ml. of chilled reagent to each tube and heat for 30 minutes at 100°. Cool and dilute to 10 ml. with concentrated sulfuric acid. Read at wave lengths from 500 mμ to 560 mμ within 1 hour. Calculate tartronic acid as follows:

$$x = s\,(\mathrm{AD} - \mathrm{D} + c)\,(\mathrm{AS} - \mathrm{S} + c)$$

in which

x = the weight of tartronic acid in the sample of dialyzate taken for analysis
s = the weight of the standard tartronic acid
c = the algebraic sum of $c_{\mathrm{K_2CO_3}} + c_{\mathrm{TA}}$.

To determine $c_{\mathrm{K_2CO_3}}$, neutralize 1 ml. of 1:11 sulfuric acid with 1 ml. of 21% potassium carbonate solution. Evaporate to dryness and heat the residue 30 minutes with 5 ml. of 2,7-dihydroxynaphthalene reagent. Similarly heat a 5-ml. reagent blank and dilute to 10 ml. with concentrated sulfuric acid. Read the increased absorbance due to potassium carbonate at the wave length of interest. This is $c_{\mathrm{K_2CO_3}}$. To determine c_{TA} measure the effect produced at the wave length used for the sample by 0.001-0.04 mg. of tartronic acid using the method described for potassium carbonate. Apply a correction for lead tartronate as 0.01 mg. per ml. of the supernatant liquid from which lead tartronate was precipitated. Thus x corrected = (0.01 × volume of the supernatant liquid from the lead precipitation) + weight of tartronic acid found in the sample in mg.

MALIC ACID

Malic acid heated with sulfuric acid in the presence of 2,7-naphthalenediol produces a pale yellow color which has a strong absorption peak at 385 mμ.[10] A probable mechanism for the reaction involves the formation of malonaldehyde which condenses with the reagent to form hydroxynaphthalene-α-pyrone. Succinic and malic acids are separated from citric acid by a strongly basic anion-exchange resin in the carbonate form rather than in the hydroxyl form. The latter takes up reducing sugars with subsequent production of interfering hydroxy acids.

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[10] A. E. Goodban and J. B. Stark, *Anal. Chem.* **29**, 283-7 (1957).

Since there is a slow production of formaldehyde during the elution step, the operation of the column should not be interrupted during the collection of the malic acid fraction. A correction factor must be applied for tartaric and α-ketoglutaric acids. 2-Ketoglutaric acid interferes. Beer's law is followed. 2,4-Dinitrophenylhydrazine is also a reagent for malic acid.[11] (Vol. III, p. 289.)

Sample—*Plant material.* Mix a 1-gram sample with 5 grams of anhydrous sodium sulfate, acidify with 1:10 sulfuric acid, and extract with ether. Distil off the ether and take up the residue in water. Neutralize with 0.4% sodium hydroxide solution and dilute to the desired volume. Develop a 5-ml. aliquot with 2,4-dinitrophenylhydrazine.

Procedure—*By 2,7-naphthalenediol.* Grind the Amerblite IRA 400 resin to 60-80 mesh and sieve wet in the chloride form. Regenerate with 5% sodium hydroxide solution and wash with 6.2% sodium carbonate solution until converted to the carbonate form. Prepare a borosilicate glass column 14 mm. × 20 cm., closed at one end with a rubber stopper. Use a disc of nylon bolting cloth to retain the resin in place. Load the column with the water-slurry of the carbonate form of the basic anion-exchange resin so that the volume after settling is 10 ml. Fill a second column to a volume of 10 ml. with Dowex 50, hydrogen form, 60-100 mesh, cross linkage 12%. Support the second column above the first so that the effluent drips directly into the anion column. Stopcocks are not necessary as the columns will not drain dry if the resin is ground to 60-80 mesh.

Carefully add an aliquot containing up to 3 milliequivalents of total acid and at least 0.008 milliequivalent of malic acid to the upper column and allow it to run freely through both columns. Wash the upper column with three 10-ml. portions of water, allowing each portion to drain. Remove the upper column and wash the lower column with three 10-ml. portions of water. If no determinations of lactic or glycolic acids are to be made, the second series of washings can be eliminated. Elute the lower column with 50 ml. of 1.425% ammonium carbonate solution in 10-ml. portions. This eluate contains all of the lactic, glycolic, and glyceric acids. Methods for determining them appear respectively at pages 329, 315, and 332. Wash the column with 49 ml. of 5.7% ammonium carbonate solution and collect the eluate. Dilute the eluate to 50 ml. with water. It contains the malic acid.

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[11] I. Reifer, *Bull. acad. polon. sci. Class II,* 3, 207-10 (1955).

Carefully add 6 ml. of concentrated sulfuric acid to a 1-ml. aliquot of the eluate containing 0.005-0.08 mg. of malic acid down the side of the tube to avoid rapid evolution of carbon dioxide. Add 0.1 ml. of 1% 2,7-naphthalenediol solution in concentrated sulfuric acid. Mix and heat at 100° for 20 minutes. Cool and read at 390 mμ. If tartaric and α-ketoglutaric acids are present also read at 490 mμ. Then apply the following correction factor for tartaric and α-ketoglutaric acids:

$$A_{390\ (\text{malic})} = A_{390\ (\text{total})} - \frac{A_{490\ (\text{total})}}{2}.$$

By 2,4-dinitrophenylhydrazine. Oxidize a 5-ml. sample containing 0.5-16 mg. of malic acid with 0.5 ml. of 1.2% potassium bromide solution and 0.5 ml. of 0.6% potassium permanganate solution in the presence of 1 ml. of 1:1 sulfuric acid. After 15 minutes at 20°, cool in ice and remove excess oxidizing agents with a few drops of 5% sodium sulfite solution. Heat at 100° with 1 ml. of 0.05% 2,4-dinitrophenylhydrazine solution for 15 minutes and dilute to 20 ml. Shake with 3 ml. of benzene. Wash the extract twice with 2 ml. of 1:1 sulfuric acid and 3 times with 5-ml. portions of water. Add 0.5 gram of anhydrous sodium carbonate and 3 ml. of 1% sodium hydroxide solution in 95% ethanol. Shake, filter through cotton wool, and read against a blank prepared in the same way as the sample with the exception that the sulfuric acid is added after the potassium permanganate is removed with the sodium sulfite.

Citric Acid

Citric acid is quantitatively converted to pentabromoacetone by oxidation in the presence of bromine with a vanadate oxidizing reagent as well as the common reagent, potassium permanganate. With the vanadate oxidizing reagent a yellow color is developed with sodium sulfide[12] (Vol. III, p. 394), a yellow color with thiourea[13] (Vol. III, p. 394), or a pink color with pyridine in the presence of potassium hydroxide.[14] The pentabromoacetone is extracted with *n*-heptane prior to color development.

A modification of the usual pentabromoacetone reaction, using a potassium permanganate oxidizing agent, carries out the oxidation and bromination in the presence of chloroform which extracts the pentabromoacetone as it is formed protecting it from the effects of excess oxidizing agents.[15] In this technic, the color is developed in alkaline solution with resorcinol and ammonium sulfate. With sodium sulfide, glycerol may be added as a stabilizer against bright daylight or sunlight, although it is not needed if the color is read within 15 minutes away from bright light.[16]

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[12] T. G. Taylor, *Biochem. J.* **54**, 48-9 (1953).

[13] B. McArdle, *Ibid.* **60**, 647-9 (1955); E. Beutler and M. Yeh, *J. Lab. Clin. Med.* **54**, 125-31 (1959).

[14] W. C. Hess, *J. Dental Research* **34**, 462-5 (1955).

Ferric chloride in the presence of hydrochloric acid is a reagent for citric acid in deproteinized samples. The color is stable for 10 minutes and Beer's law is followed up to 15 mg.[17]

Procedure—*As pentabromoacetone. By vanadate and sodium sulfide.*[18] As a bromide-bromate-vanadate reagent, dissolve 19.836 grams of potassium bromide, 5.4 grams of potassium bromate, and 12 grams of vanadic acid in water and dilute to 1 liter.

To a 5-ml. aliquot of sample containing 0.02-0.2 mg. of citric acid add 5 ml. of 3:1 sulfuric acid. Mix and add 5 ml. of reagent. After 20-30 minutes, add 2 ml. of 22% ferrous sulfate heptahydrate solution in 1:35 sulfuric acid kept under hydrogen and let stand for 10 minutes. The solution turns emerald green when all bromine released in the reagent has been destroyed. Shake for 1 minute with hexane and remove the lower aqueous layer. Add sufficient anhydrous sodium sulfate to absorb the acid and dehydrate the hexane. Shake 5 ml. with 3 ml. of fresh 2% sodium sulfide solution. Remove the yellow aqueous layer and filter. Read a 450 mμ.

By vanadate and thiourea.[19] *Biological fluids.* Mix a 5-ml. sample with 5 ml. of concentrated sulfuric acid. After 10 minutes, add 5 ml. of the reagent specified for the immediately preceding method. Mix, heat at 30 for 20 minutes, and add 3 ml. of 22% ferrous sulfate heptahydrate solution. Mix and shake for 2-2.5 minutes with 6.5 ml. of hexane. Remove the lower aqueous layer and wash the petroleum-ether layer with 15 ml of water. Remove the last traces of water with anhydrous sodium sulfate To 5 ml. of the hexane extract, add 4 ml. of a solution containing 4 gram of thiourea in 100 ml. of 2% sodium tetraborate solution. Mix by passing a stream of air through for 1.5 minutes and read at 435 mμ.

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[15] I. Riefer, *Bull. acad. polon. sci. Classe III* **2**, 145-8 (1954); *Acta Biochim. Polon* **1**, 293-305 (1954).

[16] K. Taufel and H. Rutloff, *Ernahrungsforschung* **1**, 693-703 (1956).

[17] J. Storck, *Ann. biol. clin. (Paris)* **12**, 419-21 (1954).

[18] T. G. Taylor, *Biochem. J.* **54**, 58-9 (1953).

[19] B. McArdle, *Biochem. J.* **60**, 647-9 (1955).

By permanganate and resorcinol.[20] To a 5-ml. sample containing 0.0005-0.04 mg. of citric acid in the presence of 10 ml. of chloroform, add 0.3 ml. of 1:1 sulfuric acid, 0.2 ml. of 1.2% potassium bromide solution, and 1 ml. of 4.75% potassium permanganate solution. Shake. Reduce the excess permanganate with 3% hydrogen peroxide. Shake and separate the chloroform phase. Extract the aqueous phase twice with 2-ml. portions of chloroform, and evaporate the combined chloroform extracts to dryness. Take up to residue in 0.2 ml. of 1% trisodium phosphate—1% sodium carbonate buffer. Add 1 ml. of 2.5% resorcinol solution and 3.8 ml. of 5% ammonium sulfate solution. Allow the color to develop for 10 minutes in ice and read with a blue filter.

By permanganate and sodium sulfide.[21] *Blood.* Add 5 ml. of water to a 2-ml. sample, stabilized with oxalate, and dilute nearly to 15 ml. with 20% trichloroacetic acid. Mix, heat for 10 minutes at 90°, and cool. Dilute to 15 ml. with water and centrifuge.

Filter the centrifugate and to a 10-ml. aliquot, add 1 ml. of 1:1 sulfuric acid. Concentrate to 6 ml. Cool to room temperature and add 0.25 ml. of 40% saturated metaphosphoric acid solution. Add 0.5 ml. of 12% potassium bromide solution and 2 ml. of 5% potassium permanganate solution. After 10 minutes decolorize excess permanganate with 3% hydrogen peroxide. Shake with 6 ml. of hexane. Let stand for 3 minutes and remove the lower layer. Wash the hexane layer twice with 5-ml. portions of water. Add 6 ml. of 2% sodium sulfide solution, shake, and centrifuge. Read the aqueous layer at 450 mμ against sodium sulfide solution.

Plasma.[22] Add 3 drops of 4% heparin solution to whole blood immediately after drawing. Cool to 20° and centrifuge. To 3 ml. of the supernatant liquid, add 15 ml. of 10% trichloroacetic acid with continuous shaking. Filter after 10 minutes.

Heat 15 ml. of the deproteinized filtrate containing 0.05-0.1 mg. of citric acid with 2 ml. of concentrated sulfuric acid for 10 minutes at 100°. Cool to room temperature, add 1 ml. of saturated bromine water. If the yellow bromine color disappears in less than 5 minutes, add additional bromine water until the color is stable. Add 1 ml. of 12% potassium bromide solution and 4 ml. of 4.7% potassium permanganate solution with shaking. Add additional potassium permanganate solution if necessary so that the violet color is permanent for 30 minutes at room temperature. Cool to 1-2°. Remove excess bromine, potassium permanganate, and manganese dioxide by addition of cooled 3% hydrogen peroxide solution in portions at 30-second intervals. This step may require 30 minutes. Transfer the colorless solution to a separatory funnel, wash the flask with two small portions of water, and finally with 15 ml. of hexane at 1-2°. Shake the washings and solution vigorously for 1 minute. Allow the layers to separate and discard the aqueous phase. Wash the hexane with 10 ml. of water followed by 10 ml. of phosphate buffer for pH 7, shaking vigorously during the washings. Wash the stopper with hexane and carefully remove the aqueous phase. Add 10 ml. of 4% thiourea in 4% sodium tetraborate solution to the hexane layer. Shake well for 5 minutes. Centrifuge and read the aqueous layer at 450 mμ within 25 minutes.

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[20] I. Riefer, *Bull. acad. polon. sci. Classe III*, 2, 145-8 (1954); *Acta Biochim. Polon.* 1, 293-305 (1954).

[21] K. Taufel, R. Pohloudek-Fabini, and U. Behnke, *Z. anal. Chem.* 146, 244-50 (1955).

[22] K. F. Gey, *Intern. Z. Vitaminforsch.* 25, 21-41 (1953).

Whole blood. Deproteinize 5 ml. of heparinized blood with 25 ml. of 10% trichloroacetic acid solution and filter. Mix 20 ml. of the filtrate with 2.4 ml. of concentrated sulfuric acid and heat for 10 minutes at 100°. Proceed as for plasma from "Cool to room temperature, add 1 ml. . . ."

Urine. Dilute a sample 1:10. Deproteinize albuminiferous urine with 10% trichloroacetic acid solution plus water to obtain the 1:10 dilution. To 2 ml. of this dilution add 3 ml. of concentrated sulfuric acid. Heat for 10 minutes at 100° and cool to room temperature. Add 2 ml. of saturated bromine water and proceed as for plasma from "If the yellow bromine color. . . ." Increase the amount of potassium permanganate solution used to 8 ml.

Milk.[23] Treat a 20-ml. sample successively with 5 ml. of 1:1 sulfuric acid, 1 ml. of 10.6% potassium ferrocyanide solution, and 1 ml. of a solution containing 233 grams of zinc acetate dihydrate and 30 grams of glacial acetic acid per liter. Shake after addition of each reagent. Dilute to 50 ml. with water and filter. Treat a 15-ml. aliquot of the filtrate with 2 ml. of concentrated sulfuric acid at 100° for 10 minutes. Complete as for plasma from "Cool to room temperature. . . ."

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[23] R. Coppens, *Bull. inst. agron. et stas. recherches Gembloux* **17**, 16-25 (1948-9).

By ferric chloride.[24] *Blood or plasma.* To a 5-ml. sample add 15 ml. f a deproteinizing solution prepared by diluting 3 grams of mercuric hloride and 3 grams of zinc sulfate heptahydrate to 100 ml. with water. Mix and filter. To a 5-ml. aliquot of the filtrate containing 1-15 mg. of itric acid, add 4 ml. of 1:110 hydrochloric acid and 1 ml. of 6.2% ferric hloride solution. Dilute to 10 ml. Read at 445 mμ against a reagent lank.

Polycarboxylic Acids

Polycarboxylic acids form fluorescent derivatives of various colors, Table 21, when heated with resorcinol and sulfuric acid following chromatographic separation on silica gel.[25] Polycarboxylic acids with no other functional group react with resorcinol and sulfuric acid to produce compounds similar to fluorescein. Acids containing unsaturation or hydroxy groups form umbelliferones under the same conditions. Derivatives of individual acids fluoresce at different wave lengths to yield yellow-green to violet. Thus a fraction is identifiable by its shade.

TABLE 21. COLOR AND INTENSITY OF FLUORESCENCE OF SOLUTIONS OF POLYCARBOXYLIC ACIDS

Acid	*Color of solution*	*Color of fluorescence*	*Relative fluorescence,*[a] *40 γ acid with blue filter*
Fumaric	Colorless	Blue-violet	24
α-Ketoglutaric	Yellow	Blue-green	35
Oxalacetic	Orange	Blue-green	12
Succinic	Colorless	Yellow-green	20
Aconitic	Colorless	Light blue	75
Malic	Colorless	Blue-violet	22
Isocitric	Colorless	Light blue	58
Citric	Colorless	Sky blue	89

[a] The instrument was adjusted so that 0.02 mg. % of quinine sulfate read 39.

If the heating is at over 120° the blanks develop appreciable fluorescence. A substantial excess of resorcinol is necessary to avoid the need of strict control.

Procedure—To 3 grams of silicic acid, add 0.15 ml. of concentrated sulfuric acid and 1 ml. of water. Mix until smooth and pasty. Add 50 ml.

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[24] J. Storck, *Ann. biol. clin. (Paris)* 12, 419-21 (1954).

[25] C. Frohman and J. Orten, *J. Biol. Chem.* 205, 717-23 (1953).

of chloroform in 5-ml. portions with vigorous stirring between additions Pour the smooth slurry into an 8 mm. × 75 cm. tube. Pass 200 ml. o chloroform through the silica gel to pack the column.[26] Introduce 1 ml of sample solution containing 0.01-0.02 mg. of the acid in 4% tertiar amyl alcohol solution in chloroform into the column. When the level o the solution drops to that of the silica gel, add 50 ml. of chloroform. Whe the level of the liquid again reaches that of the silica gel, complete th elution with a 1.4 mixture of tertiary amyl alcohol and chloroform, collecting 2-ml. fractions. Dilute each fraction to 4 ml. with water and ad 0.1 ml. of 0.4% resorcinol solution in absolute ethanol.

Heat at 108° for 20 minutes to vaporize the solvents. Aspirate for minutes while still in the bath to remove the last traces of the tertiar amyl alcohol. After the tubes are dry add 0.1 ml. of concentrated sulfuric acid and heat at 100° for 20 minutes. Dissolve in 5 ml. of 6% sodium hydroxide solution and measure the fluorescence with a blue filte (Coleman B2). Shake, dilute with water to 50 ml., shake, and read wit a green filter.

Ethylenediaminetetraacetic Acid

The procedure of determining chromium with ethylenediaminetetraacetic acid as a complexing agent is reversed to provide a method fo determining ethylenediaminetetraacetic acid. No anions or cations nee be removed since the formation constant of chromium with the chelatin agent is sufficiently high to preclude the possibility of interference.[27] Fo reproducible results arsenious acid is used to reduce excess chromium Although hydrochloric acid, ethanol, oxalic acid, or zinc amalgam ma be substituted, the results are not readily reproducible. Beer's law is followed at 560 mμ and 395 mμ.

Ethylenediaminetetraacetic acid is determined in cleaners and preparations used to remove lime deposits by the reaction of cobalt nitrate i the presence of hydrogen peroxide.[28]

Procedure—*Urine. By chromium.* As a reducing agent, dissolve 9.89 grams of arsenious acid and 7 grams of sodium hydroxide in 300 ml. o water. Neutralize with glacial acetic acid, dilute to 600 ml., and slowl add 400 ml. of glacial acetic acid with mixing. Dilute to 1 liter. Add ml. of 0.5% sodium chromate solution to a 2-ml. sample containing 1-3 mg. of ethylenediaminetetraacetic acid per ml. of solution. Mix and add 2 ml. of the arsenious acid reducing agent. Heat at 100° for 5 minutes and read at 560 mμ against a reagent blank. The blue color of reduced chromium in the blank does not interfere.

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[26] C. Frohman, J. Orten, and A. Smith, *Ibid.*, **193**, 277-83 (1951).

[27] P. J. Cherney, B. Crafts, H. H. Hagermoser, A. J. Boule, R. Harbin, and B. Zak *Anal. Chem.* **26**, 1806-9 (1954).

[28] P. Leman, *Ann. fals. et fraudes* **51**, 171-2 (1958).

By cobalt. Mix 1 ml. of a 1:10 dilution of the sample with 0.5 ml. of glacial acetic acid, 0.2 ml. of 1% cobalt nitrate solution, and 1 ml. of 30% hydrogen peroxide. Heat for 15 minutes at 100°, dilute to 5 ml., and read the absorbancy.

DISODIUM DIHYDROGEN ETHYLENEDIAMINETETRAACETATE

The complexing ability of ethylenediaminetetraacetic acid is the basis for the determination of disodium dihydrogen ethylenediaminetetraacetate with an excess of cupric ions.[29] Excess reagent is required to displace iron, calcium, and magnesium from their complexes with disodium dihydrogen ethylenediaminetetraacetate. The excess copper which precipitates as copper hydroxide is removed by filtration or centrifuging together with the hydroxides of other precipitated metals.

The complex is developed in a sodium hydrogen sulfate buffer at pH 11. A reagent buffer is required as a reference solution since the calibration curve is nonlinear if water, buffer, or sample solution are used as a reference. Beer's law is followed and the complex exhibits a maximum between 230 and 260 mμ. Nickel and chromate interfere at concentrations below one-tenth that of the sample but cobalt does not.

Procedure—To a 5-ml. sample containing 0.125-1.5 mg. of disodium dihydrogen ethylenediaminetetraacetate, add 4 ml. of 0.25% copper sulfate pentahydrate solution. Dilute to 25 ml. with 1.42% disodium hydrogen phosphate solution which has been adjusted to pH 11 with 20% sodium hydroxide solution. Filter. Prepare a reference solution by diluting 4 ml. of 0.25% copper sulfate pentahydrate solution to 25 ml. with the same buffer, and filter. Read the sample against the reference solution at 250 mμ.

KAINIC ACID, DIGENIC ACID

The reaction of kainic acid with ninhydrin produces a product which is extracted into benzene and read at 429 mμ.[30] There is interference from over twice the molar amount of amino acid or ammonium salts, but suga or inorganic salts do not interfere. An alternative is reaction with *p*-benzoquinone.[31]

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[29] O. Menis, H. P. House, and I. B. Rubin, *Anal. Chem.* **28**, 1439-41 (1956).

[30] M. Miyasaki, R. Haraoka, K. Fujisaki, and A. Morimoto, *J. Pharm. Soc. Japan* **75**, 477-9. (1955).

Procedure—*By ninhydrin.* Heat a 0.5-ml. sample solution containing 0.05 mg. of kainic acid per ml. with 0.5 ml. of a buffer solution at pH 5, 7 ml. of benzene, and 2 ml. of 2% ninhydrin solution for 5-11 minutes at 60°. Remove the benzene layer and dilute it to 10 ml. Read at 429 mμ.

By p-*benzoquinone.* To 1 ml. of sample containing 0.25 mg. of kainic acid add 2 ml. of phosphate buffer for pH 6. Add 1 ml. of 2% *p*-benzoquinone in ethanol and incubate at 30° for 40 minutes. Dilute to 10 ml with water and read at 520 mμ.

4-AMINO-N^{10}-METHYLPTEROYLGLUTAMIC ACID, AMETHOPTERIN

Amethopterin can be determined in plasma since it shows fivefold increased fluorescence upon oxidation whereas normally fluorescing compounds in plasma do not.[32]

Procedure—*Plasma.* Deproteinize 1 ml. by mixing with 9 ml. of 10% trichloroacetic acid solution. Centrifuge and adjust a 5-ml. aliquot of the centrifugate to just under pH 5 by addition of 10% sodium hydroxide solution dropwise. Dilute to 10 ml. with acetate buffer for pH 5. Read the fluorescence at 450 mμ. Add 10% potassium permanganate dropwise until an excess is present. Just decolorize by dropwise addition of 3% hydrogen peroxide solution. Read at 450 mμ and subtract the background absorption previously determined.

CIS-ACONITIC ACID

Pyridine and acetic anhydride are reagents for *cis*-aconitic acid in alkaline solution.[33]

Procedure—*Citrate absent.* Alkalize a 0.5-ml. sample with 1:1 ammonium hydroxide. Add 2 ml. of 95% ethanol and evaporate to dryness. Repeat the evaporation three times after adding in each case 1 ml. of 95% ethanol. Cool to 0°, add 3 ml. of cold 95% ethanol, and shake. Add

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[31] T. Oshima, K. Mizutani, and K. Fujisaki, Japanese patent 2950 (1958).

[32] M. V. Freeman, *J. Pharmacol. Exptl. Therap.* 120, 1-8 (1957).

[33] Jacques Wajzer, *Bull. soc. chim. biol.* 35, 695-6 (1953).

0.5 ml. of a 7:3 mixture of pyridine and acetic anhydride. Read after 10 minutes at 370 mμ.

Citrate present. Proceed as above through the evaporations with ethanol. Add 0.5 ml. of a 7:3 mixture of pyridine and acetic anhydride. Stopper and heat at 100° for 10 minutes. Cool, add 3 ml. of 95% ethanol, and read within 10 minutes at 370 mμ.

PECTIN

Pectin can be classified as a polygalacturonic acid. For determination it is hydrolyzed in alkaline solution to pectic acid. The latter is then determined by reaction with carbazole[34] (Vol. III, pp. 221-2). Fats are removed from the sample by extraction with petroleum ether or diethyl ether. Color and tannins are extracted with acidified ethanol.

A photometric method for soluble pectin, polygalacturonic acids, uses sulfuric acid as a reagent. The interference of starch is eliminated by centrifuging the sample and the interference of protein by boiling. The pectin is precipitated by acidified ethanol.[35] The period of heating is not critical. If the sample is unripe apples, nongalacturonic acid materials are also included in the analysis. Galacturonic acid is used to prepare the standard curve.

The acetyl content of pectin is also determined. Pectin hydroxamic acids form an insoluble complex with ferric iron while acetohydroxamic acid, produced from the secondary acetyl groups of pectin, forms a soluble red complex. These reactions serve as the basis of determination of 0.086-0.43 mg. of acetyl per ml. of pectin substances and 2-11 mg. of acetyl in acetylated carbohydrate polymers.[36] The sample is added to the hydroxylamine reagent to ensure the presence of excess reagent at all times. The order of addition of reagents is important since the formation of hydroxamic acids at reduced rate was found even in acidic conditions and sodium hydroxide added first de-esterified the sample so that no esters were available for hydroxamic acid formation. Alcoholic solutions are used to ensure complete precipitation of the pectin hydroxamic acid-ferric complex. The soluble acetohydroxamic acid-ferric complex is stable for 1 hour and the color independent of temperature from 20-30°. The stock reagent solution is stable for 1 month under refrigeration and the reagent solution is stable for 1 week at room temperature. Beer's law is followed.

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[34] "Official Methods of Analysis of The Association of Official Agricultural Chemists," 8th ed., pp. 195-7, Association of Official Agricultural Chemists, Washington, D. C. (1955).

[35] J. M. Lawrence and K. Groves, *J. Agr. Food Chem.* **2**, 882-5 (1954); V. Kutz, Mrs. V. Kutz, and J. Ott, *Élelmezési Ipar* **11**, 71-3 (1957).

[36] E. A. McComb and R. M. McCready, *Anal. Chem.* **29**, 819-21 (1957).

Samples—*Sweet chocolate containing no milk solids.* Weigh a 14-60 gram well-mixed, grated sample containing 4.7-5.2 grams of dry fat-free cacao. If necessary for manipulation divide it into equal portions. To extract the fat add 120 ml. of 30-60° petroleum ether, or 120 ml. of diethyl ether at about 30°. Shake thoroughly and centrifuge to extract the fat. Repeat the extraction with another 100-ml. portion of solvent and finally with 100 ml. of ethanol. Discard the extracts.

To extract color and tannins, add 150 ml. of acidified ethanol prepared by diluting 10 ml. of concentrated hydrochloric acid and 432 ml. of 95% ethanol to 500 ml. with water. Heat to 55°, stopper, and shake vigorously for 2 minutes. Centrifuge 6-8 minutes and discard the supernatant liquid. Add 100 ml. of 95% ethanol. Shake, centrifuge, and discard the supernatant liquid.

Add 150 ml. of water and shake vigorously to disperse the residue. Make the mixture alkaline to litmus with 1:1 ammonium hydroxide, noting the amount used. This will usually be about 1.4 ml. Acidify with glacial acetic acid, adding 0.5 ml. in excess. Add 50 ml. of 2% ammonium oxalate monohydrate solution. Stir for 3 hours, maintaining the solution at 90-92° while stirring. If the level of the solution is reduced appreciably, add hot water to restore the original volume. Cool to 45° and dilute quantitatively to 250 ml. with water at 45°, adding 1.5 ml. in excess to correct for the volume of cacao solids. Mix well and centrifuge for 15 minutes. If desired, reserve the cacao residue for the estimation of fat-free cacao.

Warm the supernatant liquid, which may be turbid or opalescent, to 45° and measure the volume. Cool to 15-17° and make alkaline to phenolphthalein with 15% sodium hydroxide solution, adding 11 ml. in excess. If more than 1.15 ml. of ammonium hydroxide was added previously, add more than 11 ml. of sodium hydroxide solution to displace the ammonia. Stir and let stand for 20 minutes at 15-17°. Decant the alkaline liquid and divide into two parts. Rinse the original container with 5-8 ml. of cold water.

Add with stirring 10 ml. of concentrated hydrochloric acid, 40 ml. of 95% ethanol, and 0.8-1 gram of a 1:1 mixture of Filter-Cel and Celite. Stopper, shake well, and centrifuge for 10-12 minutes. Decant and wash the residue by shaking with 100 ml. of 95% ethanol. Centrifuge and discard the supernatant liquid.

Add 75 ml. of water and shake. Make slightly alkaline to litmus with a few drops of 1:1 ammonium hydroxide and add 0.5 ml. in excess. Stopper and shake thoroughly 1-1.5 minutes to dissolve the pectic acid precipitate. Filter with suction and rinse the container with two 25-ml. portions of water, containing 1-2 drops of 1:1 ammonium hydroxide, adding each separately to the precipitate and filtering with suction.

Transfer the filtrate, rinsing the flask twice with 5-ml. portions of water. Add 5 ml. of concentrated hydrochloric acid and 90-100 ml. of 95% ethanol. Stopper, shake, and centrifuge for 8 minutes. Filter the supernatant liquid on a fluted paper and add the residue to the filter paper, draining thoroughly. Do not rinse. Take up the residue remaining in the centrifuge tube and the residue on the filter paper with a total of 75 ml. of water at 60-75°. Cool to 15-17° and add with stirring cooled 15% sodium hydroxide solution until the mixture is alkaline to phenolphthalein. Add 3 ml. in excess and let stand at 15-17° for 15 minutes. Acidify with 10 ml. of 2:5 hydrochloric acid and dilute to 100 ml. with water. Add a few glass beads, cover and boil for 5 minutes. Add 10 ml. of concentrated hydrochloric acid with stirring, and 400 mg. of alkali- and acid-washed and ignited asbestos. Stir for 40 seconds, and immediately filter with gentle suction. The filtrate should be clear or slightly opalescent with no immediate separation of the precipitate. Wash the beaker with three 25-ml. portions of a mixture of 200 ml. of water, 50 ml. of 95% ethanol, and 20 ml. of 2:5 hydrochloric acid which has been warmed to 100° and add to the filter. Wash the beaker and filter with four to five 25-ml. portions of a solution containing 400 ml. of 95% ethanol diluted to 950 ml. with water and warmed to 100°. Washings should be clear and pass through the filter readily. Ignore the appearance of a precipitate in the flask.

Take up the washed pectic acid with 100 ml. of water and make slightly alkaline to phenolphthalein with 4% sodium hydroxide solution, adding 5 ml. in excess. Shake for 2 minutes, filter, and wash 4-5 times with water. Nearly neutralize the alkaline filtrate and dilute to 1 liter.[37] Develop by carbazole.

Chocolate liquor, breakfast cocoa, cocoa, and low-fat cocoa. Mix powdered samples thoroughly and take a 15-gram sample. Chill chocolate liquor samples until hard and grate or shave a 25-gram sample. Shake the sample with 100 ml. of 30-65° petroleum ether or diethyl ether. Centrifuge and repeat the extraction of fat with two additional 100-ml. portions of solvent. Filter with moderate suction. Allow the residue to suck dry and grind gently to pulverize. Mix and dry approximately 45 minutes in a covered aluminum dish at 100°. Cool in a desiccator. Extract a 5-gram sample with 100 ml. of 95% ethanol. Discard the supernatant liquid and continue following the procedure under sweet chocolate containing no milk solids starting with "Add 150 ml. of water and shake vigorously to disperse the residue. . . ."

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[37] H. Shuman and W. O. Winkler, *J. Assoc. Offic. Agr. Chemists* 38, 605-7 (1955).

Cacao products containing milk solids. Weigh a 60-110 gram sample containing 5 grams of fat-free liquor and distribute equally between two centrifuge bottles. Shake each portion with 120 ml. of petroleum ether or diethyl ether at 25-30°. Centrifuge and decant the supernatant liquid. Repeat the extraction of fat with one 120-ml. portion of solvent and then with a 100-110 ml. portion of acetone. To each residue add approximately 90 ml. of acetone or enough acetone to make the volume 110 ml. assuming that one-half of a 75-gram sample retains 20 ml. of acetone. Stopper and shake. To extract milk proteins, add 100 ml. of 18% triethanolamine solution to each. Stopper and shake for 2 minutes. Allow the foam to subside for 1 minute and centrifuge for 12-14 minutes. Extract the residues with 100-120 ml. portions of 11:10 acetone—18% triethanolamine solution. Centrifuge and decant. To each residue add 100 ml. of 85% ethanol. Shake, centrifuge, and discard the supernatant liquid. To each residue add 15-20 ml. of acidified 82% ethanol prepared by diluting 10 ml. of concentrated hydrochloric acid and 432 ml. of absolute ethanol to 500 ml. with water. Stir and make the residue acid to litmus with 1:1 hydrochloric acid. Follow the procedure for sweet chocolate starting with "To extract color and tannins. . . ."

Apples. Freeze, thaw, peel, core, and put the sample through a food grinder. Homogenize to break up cell structure. Centrifuge in a basket centrifuge with filter cloth over the holes to avoid starch interference. Return an early portion to the centrifuge and recentrifuge. Boil gently for 15 minutes to precipitate protein and to inactivate enzymes. Replace any evaporated water, centrifuge for 10 minutes, and decant. To a 10-ml. aliquot add slowly 1 ml. of 1:6 sulfuric acid and 40 ml. of 95% ethanol with stirring. Allow 30 minutes for pectin to precipitate and centrifuge for 10 minutes. Wash the precipitate twice by suspension in acidified alcohol prepared by mixing 1 part of 1:6 sulfuric acid, 10 parts of water, and 40 parts of 95% ethanol. Recentrifuge, decant, and dissolve the precipitate in hot water. Cool and dilute so that the final pectin concentration is 0.02-0.1 mg. per ml. Develop with sulfuric acid.

Apple juice. Follow the procedure for apples, starting with "To a 10 ml. aliquot. . . ."

Procedure—*By carbazole.*[38] Chill 2 ml. of sample in ice water. Add 12 ml. of concentrated sulfuric acid slowly and carefully. Heat at 100° for 20 minutes and cool. Add 1 ml. of 0.1% carbazole in absolute ethanol and read at 520 mμ against a sample blank after 2 hours.

By sulfuric acid. Layer 9 ml. of 4:1 sulfuric acid below 1 ml. of a sample containing 0.02-0.1 mg. of pectin. Mix and heat at 100° for 15 minutes. Cool and read at 295 mμ against a reagent blank.

As hydroxamic acids. Acetyl groups in pectic substances. To prepare the stock solution of the reagent, dissolve 1.93 grams of ferric chloride hexahydrate in 5 ml. of concentrated hydrochloric acid and add 5 ml. of 70% perchloric acid solution. Evaporate to near dryness, dilute to 100 ml. with water, and store. To 60 ml. of the stock solution add 8.3 ml. of 70% perchloric acid, cool in ice, and dilute to 500 ml. with chilled absolute methanol. To a mixture of 1 ml. of 9.5% sodium hydroxide solution and 1 ml. of 3.75% hydroxylamine hydrochloride solution, add with agitation 5 ml. of an aliquot containing 0.1-0.45 mg. of acetyl. After 5 minutes, add 5 ml. of chilled acid methanol solution containing 35.2 ml. of 70% perchloric acid per 500 ml. of absolute methanol to aid in precipitating the insoluble pectic hydroxamic acid-ferric complex. Mix and slowly add ferric perchlorate solution, mixing after each addition until a volume of 25 ml. is obtained. After 5 minutes, filter off the pectin hydroxamic acid-ferric complex and read the ferric acetohydroxamic acid complex at 520 mμ.

Acetylated carbohydrate polymers. To a sample containing 2-11 mg. of acetyl, add with stirring 25 ml. of 3.75% hydroxylamine hydrochloride solution. Add 25 ml. of 8.5% sodium hydroxide solution dropwise over a 15-minute interval. Continue stirring until the sample dissolves. To a 2-ml. aliquot add 5 ml. of water and 5 ml. of chilled acid methanol solution containing 35.2 ml. of 70% perchloric acid in 500 ml. of absolute methanol. Dilute to 25-ml. with ferric perchlorate reagent as prepared for pectic substances added in small amounts while stirring. Read at 520 mμ after 5 minutes.

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[38] E. A. McComb and R. M. McCready, *Anal. Chem.* 24, 1630-2 (1952).

CHAPTER 16

CYCLIC ACIDS, THEIR ESTERS, AND THEIR ANHYDRIDES

m-Hydroxybenzoic acid is read directly in the ultraviolet with corrections for the ortho and para acids as described for the ortho isomer, salicylic acid, page 389. The same technic at the same place applies to the para isomer. The maximum absorption of *p*-hydroxybenzoic acid with titanium sulfate is at 390 mμ, that of phenylsalicylate with the reagent at 435 mμ.

For determination of salicylsalicylic acid, it is saponified and the salicylic acid determined by ferric ion (page 388). The same technic is applied to methyl salicylate, to succinylsalicylic acid, and to phenylsalicylic acid. *N*-Butylsalicylic acid is read in the presence of benzyl benzoate in the ultraviolet, page 381.

Because of its close relation to salicylic acid, acetylsalicylic acid or aspirin is included under that topic. It is determined in aspirin-phenacetin-caffeine tablets, page 389, or read in chloroform along with salicylic acid, page 385.

A violet color of Millon's reagent (Vol. III, p. 118) and *N*-acetyl-*p*-aminosalicylic acid is extractable with butanol.[1] The maximum absorption at 565 mμ conforms to Beer's law at 0.025-1 mag. per ml. There is no interference by *p*-aminosalicylic acid, tyrosine, *o*-, *m*-, and *p*-aminophenol, *m*-amidobenzoic acid, benzoic acid, salicylic acid, anthranilic acid, and thymol.

In determination of phthalic acid in the anhydride provision is made for also reading the latter, page 400.

Various aromatic esters determinable as hydroxamic acids, page 316, are ethyl benzoate, methyl-*o*-methoxybenzoate, *n*-butyl-*o*-methoxybenzoate, methyl-*p*-hydroxybenzoate, methyl salicylate, *n*-butyl salicylate, monomethyl phthalate, mono-*n*-butylphthalate, methyl-*o*-toluate, methyl-*m*-toluate, methyl-*p*-toluate, methyl anthranilate, methyl-*p*-aminobenzoate, and acetylsalicylic acid.

Benzoic Acid

Benzoic acid can be measured in alkyd resins by ultraviolet photometry after removal of the dicarboxylic and fatty acids.[2] Carbon tetrachloride proves to have sufficiently low solvent power to separate fatty acids and benzoic acid by extraction of the former from an aqueous solution. Then the aqueous solution contains noninterfering polyhydric alcohols and potassium chloride. A correction factor of 0.97 is applied to correct for a slightly higher absorbance after benzoic acid has been carried through the procedure.

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[1] Motohiro Tamada, *Osaka Daigaku Igaku Zasshi* 9, 295-7 (1957).

[2] M. H. Swann, M. L. Adams, and D. J. Weil, *Anal. Chem.* 28, 72-3 (1956)

Both salicylic acid and benzoic acid in ether solution absorb strongly at 227 mμ. At 306 mμ salicylic acid absorbs but benzoic acid does not. This is therefore the basis for a method for determination of benzoic acid alone or in the presence of salicylic acid.[3]

Benzoic acid is read in acid methanol solution at 228 and 272 mμ. Acids of conjugated dienes interfere slightly at 228 mμ but not at 272 mμ.[4] A suitable application is to extraction of benzoic acid from butter or margarine. When converted to 3,5-dinitrobenzoic acid the violet-blue in acetone-absolute ethanol is read.[5]

Both hippuric acid and benzoic acid are read at 246 mμ where there is the maximum difference and at 220 mμ where there is the minimum difference.[6] The two values permit calculation of each.

Procedure—*Alkyd resins.* Weigh a sample containing approximately 0.1 gram of benzoic acid and dissolve in 10 ml. of benzene. Add 100 ml. of 2.8% potassium hydroxide solution in absolute ethanol. In the absence of isophthalic acid, reflux for 1 hour. In the presence of isophthalic acid, heat at 45° for at least 4 hours followed by refluxing for 1 hour. Add 150 ml. of dry benzene and stopper the flask. Cool and filter through a Gooch crucible using benzene for transferring and washing the precipitate. This precipitate is appropriate for determination of the dicarboxylic acids.

Evaporate the solvents from the filtrate at 100°, adding water periodically to replace the solvents. Dilute to 300 ml. with water and neutralize with concentrated hydrochloric acid, adding a 5-ml. excess. Extract the fatty acids with three 50-ml. portions of carbon tetrachloride, adding a fourth extraction if the third extract is colored. Reserve the water layer.

Wash the combined carbon tetrachloride layers in 50-ml. portions through three successive separatory funnels each containing 300 ml. of water. Add the 900 ml. of wash water to the water layer and dilute to 2 liters. The carbon tetrachloride may be filtered and evaporated for the determination of fatty acids, page 297. Allow a 500-ml. aliquot to stand. If further separation occurs, draw off the lower layer together with some of the aqueous layer. Filter 200 ml. of the remaining aqueous solution rapidly, discarding the first 50 ml. of filtrate. Neutralize a 100-ml. aliquot of the filtrate with 11.2% potassium hydroxide solution, adding 20 drops in excess. Evaporate to dryness using an oil bath at 110° for the final stages of evaporation. Dry in an oven at 110° for 15 minutes. Dissolve the residue in 25 ml. of water, cool, and neutralize with 1:1 hydrochloric acid, adding 1 ml. in excess. Transfer the acidified solution, rinse the beaker with 25 ml. of water followed by methanol, and dilute to 100 ml. with methanol.

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[3] D. T. Englis, B. B. Burnett, R. A. Schreiber, and J. W. Miles, *J. Agr. Food Chem.* 3, 964-9 (1955).

[4] J. B. Roos and A. Versnel, *Chem. Weekblad* 55, 67-9 (1959).

[5] P. Spanyár, E. Kever, and M. Kiszel, *Z. Lebensm.-Untersuch. u. -Forsch.* 107, 118-24 (1958).

[6] H. P. Rieder, *Clin. Chim. Acta* 2, 497-501 (1957).

Read at 273 mμ against a blank prepared by adding 1 ml. of concentrated hydrochloric acid to 50 ml. of water and diluting to 100 ml. with methanol. Unless the standard has been carried through the same procedure, that is if it is made up by dissolving benzoic acid in the blank described, multiply the result by 0.97 as a correction factor.

Ketchup. Mix 10 grams of fine salt with a 50-gram sample and make the solution basic with 10% sodium hydroxide solution. Add 2 grams of filter aid. Take up in a saturated salt solution and dilute to 1 liter with the salt solution. Mix thoroughly and let stand for 1 hour. Prepare a canvas filter cloth to fit a 15-cm. funnel by covering the cloth with a thin slurry of filter aid in water and removing the water with suction. Wash the mat gently with water and dry by drawing air through. Replace the suction flask and filter the ketchup solution with suction.

Neutralize a 100-ml. aliquot with 3:1 hydrochloric acid, adding an excess of 5 ml. of acid. Extract with 35-, 35-, 20-, and 10-ml. portions of ether, allowing the layers to separate for 5 minutes after each extraction. After the last extraction, wash the separatory funnel with 10 ml. of ether and dilute the washings and extract to 100 ml. with ether. Dilute a 10-ml. aliquot to 100 ml. with ether and read at 227 mμ for the combined absorbancies of benzoic and salicylic acids. In the absence of salicylic acid calculate as benzoic acid. In the presence of salicylic acid determine it at 306 mμ, at which wave length benzoic acid does not absorb, and apply a correction to the value at 227 mμ.

Orange-base concentrate. Follow the procedure for ketchup.

Margarine. Extract a 10-gram sample with five 40-ml. portions of 0.01% sodium hydroxide solution. To the combined extracts add 5 ml.

of alumina cream to remove suspended impurities. Dilute to 250 ml. with 0.01% sodium hydroxide solution, mix thoroughly, and filter. Dilute a 25-ml. aliquot to 100 ml. with water. Read at 224 mμ for the combined absorbancies of benzoic and salicylic acids. Read salicylic acid, if present, at 306 mμ and apply a correction at 224 mμ to determine the benzoic acid.

Alternatively, extract a 5-gram sample with 5 ml. of water and 0.5 ml. of 1:8 sulfuric acid in 60 ml. of methanol. Read at 228 or 272 mμ in methanol.

Benzyl Benzoate

The determination of the absorbancy differences between a solution containing known ingredients and one containing almost the same concentration of those ingredients is applied to the determination of benzyl benzoate and *N*-butyl acetanilide in clothing impregnant *M*-1960, which possesses repellent properties.[7] The method used differs from the differential analysis method in which the absorbancy difference between an unknown solution of high absorbancy and a reference standard of one of the components is determined at the wave length of the components' absorption peak. The same procedure is followed for the second component at the wave length of the second component. By the differential analysis method, deviations from Beer's law cannot be tolerated and the absorbancies of the constituents must be additive. The method given is less affected by these limitations. Although it is possible to determine each component simultaneously by the conventional method, the differential method provides greater accuracy. 2-Ethyl-2-butyl-1,3-propanediol and Tween 80, a sorbitol-oleic acid derivative, do not absorb in the ultraviolet from 220 to 300 mμ and therefore do not interfere.

Procedure—*Cloth.* Determine the absorbancies for 0.015% benzyl benzoate solution in 95% ethanol solution and 0.015% *N*-butyl acetanilide in 95% ethanol solution at both 255 and 280 mμ. Calculate the extinction coefficients of each substance at each wave length by dividing the absorbancies by the exact concentration in grams per liter as applying at a specified slit width. Determine the absorbancy of a 0.05% sample solution in 95% ethanol at both 255 and 280 mμ using the same slit width as used for the first two concentrations. Calculate the concentrations of *N*-butyl acetanilide and benzyl benzoate as follows:

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[7] Morton Beroza, *Anal. Chem.* **25**, 112-5 (1953).

$$k_{a280} \times C_a + k_{b280} \times C_b = A_{280}$$

$$k_{a255} \times C_a + k_{b255} \times C_b = A_{255}$$

$$\%a = \frac{C_a}{C_m} \times 100$$

$$\%b = \frac{C_b}{C_m} \times 100$$

in which

C = concentration in grams per liter
A = absorbancy of the unknown solution
k = absorbancy index or extinction coefficient
a = *N*-butyl acetanilide
b = benzyl benzoate
m = unknown mixture.

Make up a standard solution from a mixture that is prepared to contain approximately the same percentage concentration of benzyl benzoate and *N*-butyl acetanilide as determined by the above procedure. Add 2-ethyl-2-butyl-1,3-propanediol and Tween 80 in amounts that are supposed to be present. Although these components have negligible absorbance in these wave lengths they have a slight influence. Determine the differences in absorbancies between the unknown and the standard solution prepared above as follows.

Balance the instrument using the standard solution as the blank. Set the sensitivity knob about two turns from the counterclockwise limit and record the slit width. Replace the standard solution with the unknown solution and upon adjusting the density dial, obtain the absorbancy difference. If the absorbancy of the standard solution is greater than that of the unknown solution, reverse the procedure; i.e., balance the instrument with the unknown solution as the blank and read the absorbancy difference of the standard solution. Use the slit widths recorded for 255 and 280 mμ to determine the absorbancy indexes as follows.

Determine the absorbancies of the benzyl benzoate and *N*-butyl acetanilide solutions at 255 and 280 mμ against a solvent blank. Divide the absorbancies by the exact concentration in grams per liter of the solutions to obtain the absorbancy indexes. To use this procedure, Beer's law must be followed and the absorbancies of the components must be additive at the wave lengths used.

If the absorption laws are not followed, determine the absorbancy indexes as follows. Prepare a standard solution by the procedure described above, by diluting 1 gram of the mixture of the two components in the same per cent concentration as found to 100 ml. Take a 5-ml. aliquot, add 10 ml. of 0.015% *N*-butyl acetanilide solution, and dilute to 100 ml. Determine the absorbancy difference between this solution and the standard solution at each of two wave lengths. Divide the absorbancy difference at each wave length by the exact concentration of added *N*-butyl acetanilide to obtain the absorbancy index at each wave length. Determine the absorbancy index for benzyl benzoate in the same manner.

Calculate percentage composition of the two components as follows.

$$C_a = \Delta C_a + C_{as}$$

$$C_b = \Delta C_b + C_{bs}$$

$$\%a = \frac{C_a}{C_m} \times 100$$

$$\%b = \frac{C_b}{C_m} \times 100$$

ΔC = concentration difference in grams per liter

s = standard solution

$$\Delta C_a = (0.3143 \times \Delta A_{255}) - (0.3963 \times \Delta A_{280})$$

$$\Delta C_b = (0.2939 \times \Delta A_{280}) - (0.0084 \times \Delta A_{255})$$

in which

ΔA = absorbancy difference.

The above equations assume a slit width of 0.45 mm. at 280 mμ and 1.33 mm. at 255 mμ.

p-TERT-BUTYLBENZOIC ACID

p-Tert-butylbenzoic acid, separated from alkyd resins along with fatty acids, is determined in the presence of fatty acids by reading in cyclohexane at 282.5 mμ and 300 mμ.[8] At 300 mμ the *p*-tert-butylbenzoic acid has little absorbance while the fatty acids absorb at both wave lengths. The fatty acids must be of the coconut-oil type or acids similarly slightly unsaturated as highly unsaturated fatty acids will interfere with the determination. Beer's law is followed at 282.5 mμ. A slit width of 0.15 mm. is recommended for readings at 282.5 mμ as the absorbance varies with the slit width at 282.5 mμ but not at 300 mμ. Readings must be rapid due to volatility of the cyclohexane.

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[8] P. J. Secrest and B. Kosciesza, *Ibid.* 31, 1402-4 (1959).

Sample—*Alkyd resins of the coconut-oil type.* Dissolve a sample containing 1 gram of nonvolatile resin in 10 ml. of benzene and add 100 ml. of 1.4% potassium hydroxide solution in absolute ethanol. Stopper and let stand at room temperature for 1 hour. Attach an air condenser, place in cold water, and gradually heat until gentle refluxing begins. Reflux for 1 hour.

Filter using absolute ethanol for transferring. Wash the precipitate with five 20-ml. portions of absolute ethanol to remove all monobasic acid salts. Concentrate the filtrate at 100° to 15 ml. and dilute to 300 ml. with water. Extract any unsaponifiable material with ether and discard the ether layer. Neutralize the water layer with 1:5 sulfuric acid and add a 5-ml. excess. Extract the monobasic acids with three 50-ml. portions of ether. Combine the ether extracts and wash with 20-ml. portions of water until the water washes will not turn Congo red paper blue. Evaporate the ether under an inert gas to prevent oxidation of the fatty acids. Dry and if the total amount of monobasic acids is desired, cool in a desiccator and weigh.

Procedure—*In cyclohexane.* Dissolve the monobasic acids in 40 ml. of cyclohexane and rinse the flask originally containing the acids with three 10-ml. portions of cyclohexane. Dilute the combined rinsings and solution to 100 ml. with cyclohexane and dilute a 10-ml. aliquot to 100 ml. with cyclohexane. Mix and read at 282.5 mμ and 300 mμ, using a slit width of 0.15 mm. for the 282.5 mμ reading.

Determine the absorptivity of *p*-tert-butylbenzoic acid by preparing a solution containing 10 mg. of pure *p*-tert-butylbenzoic acid and 90 mg. of technical lauric acid per 100 ml. of cyclohexane. With this solution, locate the absorption peak of *p*-tert-butylbenzoic acid at 282.5 mμ. Determine the absorbance of a cyclohexane solution containing exactly 100 mg. of *p*-tert-butylbenzoic acid per liter at 282.5 mμ and 300 mμ.

Calculate the absorptivity of *p*-tert-butylbenzoic acid as follows:

$$a_{282.5} = A_{282.5}/bc$$

$$a_{300} = A_{300}/bc$$

in which

$a_{282.5}$ = absorptivity of the acid at 282.5 mμ
$A_{282.5}$ = measured absorbance at 282.5 mμ
a_{300} = absorptivity at 300 mμ
A_{300} = measured absorbance at 300 mμ
b = cell length in centimeters
c = concentration of acid in grams per liter.

Calculate the % of *p*-tert-butylbenzoic acid in the sample as follows:

% *p*-tert-butylbenzoic acid

$$= \frac{(A_{282.5} - A_{300})\ 100}{(a_{282.5} - a_{300})\ (\text{weight in grams of nonvolatile resin}) \times b}$$

in which

$A_{282.5}$ = absorbance at 282.5 mμ of *p*-tert-butylbenzoic acid in cyclohexane after extraction from sample

A_{300} = absorbance at 300 mμ of acid in cyclohexane after extraction from the sample

$a_{282.5}$ and a_{300} = absorptivities as calculated above

b = cell length in centimeters.

o-HYDROXYBENZOIC ACID, SALICYLIC ACID

The violet color produced by salicylic acid and the ferric ion (Vol. III, p. 405) varies with pH.[9] The maximum color develops at pH 3.5. Solutions over pH 6.5 fade rapidly. A buffer is not necessary unless a large excess of basic material is present. Ascorbic acid interferes.[10] The salicylic acid may be extracted with chloroform prior to color development.[11] This is applicable both to biological samples and to mixed tablets. Ether is also an appropriate extraction medium.[12] Codeine, barbiturates. and antihistamines do not interfere. In place of ferric nitrate, ferric alum has been used as a source of ferric ion.[13] Aspirin samples hydrolyze at a determined rate during the time of determination. Therefore a calculated correction is either added to the standard or subtracted from the salicylic acid found present. The method is suitable for the determination of up to 0.25% of free salicylic acid in crystalline acetylsalicylic acid or to granular or tableted starch-aspirin formulations.

Salicylic acid and acetylsalicylic acid are determined simultaneously in chloroform since acetylsalicylic acid shows a negligible absorbance at 308 mμ, and a maximum absorbance at 278 mμ.[14] Beer's law is followed and the procedure is accurate to ±0.2%. Chloroform-soluble coloring matter interferes.

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[9] R. E. Pankratz and F. J. Bandelin, *J. Am. Pharm. Assoc.* 41, 267-70 (1952); Cf. John B. Wilson, *J. Assoc. Offic. Agr. Chemists* 42, 696-8 (1959).

[10] Stanislaw Bober, *Polskie Arch. Med. Wewnętrznej* 28, 1307-10 (1958).

[11] C. B. Camponova and I. Maldonodo-Allende, *Prensa Med. argentina* 35, 1947-50 (1948); E. B. Truitt, Jr., A. M. Morgan, and J. M. Little, *J. Am. Pharm. Assoc.* 44, 142-8 (1955); R. E. Pankratz and F. J. Bandelin, *Ibid* 45, 364-6 (1956).

[12] G. Grinschgl and O. Eichorn, *Arch. Intern. pharmacodynamie* 95, 172-83 (1953).

[13] C. W. Strode, Jr., F. N. Stewart, H. O. Schott, and O. J. Coleman, *Anal. Chem.* 29, 1184-6 (1957).

[14] R. B. Tinker and A. J. McBay, *J. Am. Pharm. Assoc.* 43, 315-6 (1954); M. A. Chirigos and S. Udenfriend, *J. Lab. Clin. Med.* 54, 769-72 (1959).

A determination of both salicylic acid and *m*-hydroxybenzoic acid is possible since the ortho isomer is in the fluorescent form at pH 5.5 while the meta isomer is not.[15] Both are fluorescent at pH 12. Beer's law is followed for 0-12 ppm. with a relative error of less than 5%. *p*-Hydroxybenzoic acid is not fluorescent under the conditions of the method and therefore does not interfere.

Aspirin, phenacetin, and caffeine have maximum absorption for direct reading in chloroform at 278 mμ, 250 mμ, and 275 mμ respectively.[16] All conform to Beer's law. There is no interference by codeine phosphate, metapyrilene hydrochloride, hyoscyamine sulfate, phenyloxamine, and quinine. The same three ingredients are also read in alkaline ethanol solution by direct reading.[17]

Salicylic acid is determined by orcinol with either ferric or cupric ion,[18] or by diphenylamine.[19] The amount of salicylic acid present with benzoic acid is also determined by direct reading, page 379. The fluorescence of salicylic acid activated at 310 mμ and pH 11 is at 435 mμ.[20]

Procedure—*By ferric nitrate.*[21] *Sodium salicylate.* Dissolve a 0.5-gram sample in water and dilute to 200 ml. Dilute a 10-ml. aliquot to 1 liter. To 25 ml. of this sample solution containing 0.125-1.125 mg. of salicylic acid per ml., add 5 ml. of 1% ferric nitrate solution in 1:99 nitric acid, and dilute to 50 ml. Read at 525 mμ for interpretation from a salicylic acid curve.

As an alternative obtain the values for portions of 5 to 45 ml. of sample to get the slope of the curve. Compare that with the values in Table 22 to get the determined purity.

Salicylsalicylic acid. Dissolve a 0.25-gram sample in 25 ml. of 95% ethanol and add 25 ml. of fresh 10% potassium hydroxide solution. Stopper loosely and heat at 100° for 2 hours. Cool, add 5 ml. of concentrated hydrochloric acid, and dilute to 200 ml. with water. Dilute a

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[15] G. A. Thommes and E. Leininger, *Anal Chem.* 30, 1361-3 (1958).

[16] Arias C. Josefina, *Anales fac. farm. y bioquim., Univ. nacl. mayor San Marcos (Lima)* 6, 519-23 (1955).

[17] Hanna Bukowska, *Chem. Anal. (Warsaw)* 4, 105-12 (1959).

[18] Lars Svennerholm, *Arkiv. Kemi* 10, 577-96 (1957).

[19] A. Saifer and H. A. Siegel, *J. Lab. Clin. Med.* 53, 414-83 (1959).

[20] S. Udenfriend, D. E. Duggan, B. M. Vasta, and B. B. Brodie, *J. Pharmacol. Exptl. Therap.* 120, 26-32 (1957).

[21] R. E. Pankratz and F. J. Bandelin, *J. Am. Pharm. Assoc.* 41, 267-70 (1952).

TABLE 22. VALUES FOR THE SLOPE OF SALICYLATE CURVES DEVELOPED WITH FERRIC NITRATE

Salicylate	*Mol. wt.*	*Salicylic acid, %*	*Slope*	
			Calcd.	*Found*
Salicylic acid	138.05	100	. . .	71.43
Sodium salicylate	160.04	86.26	82.81	83.03
Salicylsalicylic acid	258.08	106.98	66.78	66.67
Succinyl salicylate	358.12	77.11	92.62	92.31
Acetylsalicylic acid	180.06	76.67	93.17	93.17
Phenyl salicylate	214.08	64.49	110.80	110.77
Methyl salicylate	152.14	90.74	78.73	79.13

10-ml. aliquot to 500 ml. and develop as described for sodium salicylate starting at "To 25 ml. of this sample solution. . . ."

Succinylsalicylic acid. Follow the procedure for salicylsalicylic acid.

Acetylsalicylic acid. Follow the procedure for salicylsalicylic acid using a 0.75-gram sample.

Phenylsalicylic acid. Follow the procedure for salicylsalicylic acid using a 0.4-gram sample.

Methyl salicylate. Dissolve a 0.5-gram sample in 25 ml. of 95% ethanol. Add 25 ml. of water and 2.5 grams of potassium hydroxide. Heat at 100° for 2 hours, cool, and dilute to 200 ml. Dilute a 10-ml. aliquot to 1 liter with water and develop a portion as described for sodium salicylate starting at "To 25 ml. of this sample solution. . . ."

Sodium salicylate ampules. Dilute 5 ml. of a solution containing 2 grams of sodium salicylate to 250 ml. and dilute a 5-ml. aliquot of this to 500 ml. Develop a portion as described for sodium salicylate starting at "To 25 ml. of this sample solution. . . ."

Theobromine-sodium salicylate tablets. Grind 20 tablets to a fine powder and dissolve a sample containing 0.3 gram of salicylic acid in water. Dilute to 500 ml. Dilute a 15-ml. aliquot to 500 ml. Develop as described for sodium salicylate starting at "To 25 ml. of this sample solution. . . ."

Aspirin-phenacetin-caffeine tablets. Grind 20 tablets to a fine powder. Weigh a portion corresponding to about 0.3 gram of salicylic acid. Dissolve in 25 ml. of 95% ethanol and complete as for salicylsalicylic acid from ". . . add 25 ml. of fresh 10% potassium hydroxide solution."

The aspirin can also be extracted from such tablets for saponification and determination as salicylic acid.[22] Shake a sample containing approximately 1 gram of acetylsalicylic acid with 25 ml. of 5% sodium bicarbonate solution. Extract with three 25-ml. portions of chloroform, washing each chloroform extract with 15-ml. portions of 5% sodium bicarbonate solution followed with 25 ml. of water. Discard the chloroform and combine the aqueous extracts. Add 50 ml. of 5% potassium hydroxide solution in 95% ethanol and heat at 100° for 2 hours. Cool, add 5 ml. of concentrated hydrochloric acid, and dilute to 250 ml. with water. Dilute a 20-ml. aliquot to 500 ml. and develop a 25-ml. portion as described under sodium salicylate starting at "To 25 ml. of this sample solution. . . ."

Salicylic acid by ferric alum. Aspirin.[23] In order to minimize the time during which hydrolysis takes place, adjust the photometer, after a 10-minute warm-up period, to 100% transmittance at 515 mμ or 575 mμ against a reagent blank. This should be freshly prepared to contain 1.9 ml. of SD 30 ethanol, 0.5 ml. of glacial acetic acid, and 2 ml. of the dilute ferric alum solution described below and be diluted to 100 ml. with water.

To prepare the ferric alum solution, dissolve 4 grams of ferric ammonium sulfate in 40 ml. of water. Dilute to 50 ml. and mix a 10-ml. portion with 10 ml. of 1:24 hydrochloric acid. Dilute to 500 ml. The sample should contain 1 gram of acetylsalicylic acid. Dissolve or slurry in 9.3 ml. of SD 30 ethanol. Dilute to 100 ml. with water at 25° and start a timer. If starch is present, filter immediately with suction. Treat a 20-ml. aliquot with 2 ml. of ferric alum reagent and dilute to 100 ml. with water. Read within 5 minutes at 515 mμ for white or green-tinted samples and at 575 mμ for pink-tinted samples. Calculate a hydrolysis correction as follows: the number of 15-second intervals required for the manipulation is multiplied by a hydrolysis constant of 0.001; the product is subtracted from the percentage of salicylic acid in 0.2 gram of aspirin represented by the aliquot of the sample.

The significance of temperature leading to this correction is indicated by the finding that hydrolysis causes the salicylic acid to increase over the first 5 minutes at a rate per minute of 0.0028% at 20°, 0.0036% at 25°, and 0.0054% at 30°.

Alternatively, cool a 2-ml. aliquot of the original undiluted alcohol solution to 10°, dilute to 100 ml. with water cooled to 10°, and develop with ferric alum. Compare within 30 seconds with standards.

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[22] R. E. Pankratz and F. J. Bandelin, *Ibid.* **45**, 364-6 (1956).

[23] C. W. Strode, Jr., F. N. Stewart, H. O. Schott, and O. J. Coleman, *Anal. Chem.* **29**, 1184-6 (1957).

Direct reading. Salicylic acid and aspirin.[24] Dissolve a 360-mg. sample in chloroform and dilute to 100 ml. with that solvent. Read at 308 mμ against chloroform as the primary reading for salicylic acid. Dilute 100:1 with chloroform and read at 278 mμ against chloroform as the primary reading for acetylsalicylate.

$$\text{Acetylsalicylic acid} = 1347A_{308} - 2.3A_{278}$$

$$\text{Salicylic acid} = 3.58A_{278} - 3.49A_{308}.$$

Tablets or capsules. Determine the average weight of 10 tablets or the contents of 10 capsules. Crush at least 10 tablets or mix the contents of at least 10 capsules. In either case take a sample equivalent to approximately 360 mg. of acetylsalicylic acid and dissolve with 50 ml. of chloroform. Stir thoroughly and let stand for 2 minutes or until the undissolved material settles. Filter through paper wet with chloroform, and rinse the beaker with chloroform. Dilute the rinsings and filtrate to 100 ml. with chloroform. Complete as for salicylic acid and aspirin starting at "Read at 308 mμ. . . ."

Aspirin, phenacetin, and caffeine.[25] Treat a sample containing 0.1 gram of aspirin and up to 0.1 gram of phenacetin, or 0.08 gram of phenacetin in the case of mixtures of aspirin, phenacetin, and caffeine, with 25 ml. of water. Add 5 ml. of 2.8% potassium hydroxide solution and dilute to 100 ml. with 95% ethanol. Dilute a 1-ml. aliquot to 100 ml. with 95% ethanol. Read at 250 and 295 mμ for mixtures of aspirin and phenacetin and at 250, 272, and 310 mμ for mixtures of aspirin, phenacetin, and caffeine against a solution of 2.8% potassium hydroxide solution in 100 ml. of 70% ethanol. Calculate aspirin in mixture with phenacetin as follows:

$$A_{250} \cdot 100 = 860 \text{ phenacetin} + 20 \text{ aspirin}$$

$$A_{295} \cdot 100 = 70 \text{ phenacetin} + 200 \text{ aspirin}.$$

Calculate aspirin in mixtures with phenacetin and caffeine as follows:

$$A_{250} \cdot 100 = 860 \text{ phenacetin} + 160 \text{ caffeine} + 20 \text{ aspirin}$$

$$A_{272} \cdot 100 = 210 \text{ phenacetin} + 476 \text{ caffeine} + 50 \text{ aspirin}$$

$$A_{310} \cdot 100 = 113 \text{ aspirin}.$$

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[24] R. B. Tinker and A. J. McDarz, *J. Am. Pharm. Assoc.* 43, 315-6 (1954).

[25] Hanna Bukowska, *Chem. Anal. (Warsaw)* 4, 105-12 (1959).

Free salicylic acid by ferric nitrate.[26] *Plasma or urine.* Add a 1-ml. sample of plasma or 1:10 urine, 1 ml. of water, and 0.5 ml. of 1:1 hydrochloric acid to 30 ml. of chloroform. Shake for 5 minutes at a 45° angle and centrifuge for 5 minutes to separate. Remove the supernatant aqueous layer and discard. Shake 20 ml. of the chloroform layer with 10 ml. of water and 0.25 ml. of 1% ferric nitrate solution in 1:19 nitric acid for 5 minutes at a 45° angle and centrifuge for 5 minutes. Read the aqueous phase at 540 mμ.

It is necessary to prepare the standard reference curve by using sodium salicylate added to plasma, because the extraction efficiency of chloroform for salicylic acid is less from plasma than from water. Salicylic acid is extracted equally well from water or dilute urine.

By fluorescence. Salicylic acid and m-*hydroxybenzoic acid.* Adjust the pH of one aliquot of the sample to 5.5 with acetate buffer, prepared by dissolving 4.67 grams of sodium acetate and 0.6 gram of glacial acetic acid in water and diluting to 1 liter. Prepare another aliquot at pH 12 by addition of 8% sodium hydroxide solution. Read the fluorescence intensities of each at 410 and 430 mμ under excitation by 314 mμ. The reading of the pH 5.5 solution is the concentration of salicylic acid. To determine the concentration of *m*-hydroxybenzoic acid, subtract a correction due to salicylic acid from the value at pH 12, as determined from the value at pH 5.5.

SALICYLURIC ACID

Salicyluric acid is determined by its fluorescence after extraction with ethylene dichloride. The fluorescence must be corrected for the presence of salicylic acid. The known free salicylic acid determined photometrically is converted to a fluorescence equivalent by reference to the appropriate standard curve relating extracted salicylic acid concentration to fluorescence. Similarly, a correction for plasma or urine blanks is applied.

Procedure—*Plasma.* Mix 2 ml. of plasma and 0.5 ml. of 1:1 hydrochloric acid. Shake with 30 ml. of ethylene dichloride containing 1.5% of isoamyl alcohol for 5 minutes at a 45° angle. Centrifuge for 5 minutes. Remove the aqueous layer and discard it.

As a buffer for pH 10 mix 75.4 ml. of 0.53% sodium carbonate solution with 24.6 ml. of 0.191% sodium tetraborate decahydrate solution. Add 20 ml. of the nonaqueous phase to 5 ml. of the buffer. Shake for 5 minutes and centrifuge for 5 minutes. Add 2 ml. of the aqueous buffer phase to 2 ml. of buffer and read in the fluorometer. Set the instrument so that 0.01 mg. of salicyluric acid gives a transmittance reading of 25%. Correct for free salicylic acid as separately determined.

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[26] E. B. Truitt, Jr., A. M. Morgan, and J. M. Little, *J. Am. Pharm. Assoc.* **44,** 142- (1955).

Urine. Mix 2 ml. of 1:20 urine dilution with 0.5 ml. of 1:1 hydrochloric acid. Add 1.5 grams of salt and continue as for plasma from "Shake with 30 ml. of. . . ." Use 4 ml. of buffer instead of 2 ml.

Salicyl Glucuronates

The glycuronic ester linkage in salicyl glucuronate can be hydrolyzed by concentrated hydrochloric acid in three hours. In such a three-hour period, 62% of any salicyluric acid present is hydrolyzed to salicylic acid. Salicylic acid, determined with ferric nitrate, therefore represents free salicylic acid, 62% of the salicyluric acid, and the salicyl glycuronates.[26]

Procedure—*Urine.* Hydrolyze a 0.5-ml. sample diluted 1:10 or 1:20 with 1 ml. of concentrated hydrochloric acid in a loosely stoppered tube for 3 hours at 100°. Cool rapidly and dilute to 2.5 ml. with water. Determine salicylic acid by ferric nitrate, page 386. Salicyl glycuronate is then equivalent to the total salicylic acid after hydrolysis minus free salicylic acid as separately determined and minus 0.62% of the salicyluric acid present as separately determined.

3,4-Dihydroxybenzoic Acid

3,4-Dihydroxybenzoic acid is extracted from the sample with ether and determined by ferric chloride.[27]

Procedure—*Urine.* Concentrate a sample and cool a 50-ml. aliquot. Acidify to Congo red and maintain at 0° for 4-5 hours. Dilute to 100 ml., filter, and heat at 100° for 20 minutes. Filter, dilute to 100 ml., and extract 50 ml. with ether for 24 hours. Add 10 ml. of chloroform to the ether extract, shake, and let stand for 5 minutes. Shake with 25 ml. of water and 3 ml. of 1% ferric chloride solution for 5 minutes. Separate the aqueous green phase and repeat the extraction until no more green color appears. The pH should not fall below 5. Add drops of dilute sodium bicarbonate solution if necessary. Centrifuge the ether from the aqueous phase and dilute the latter to 10 ml. with 1% ferric chloride solution. Dilute to 100 ml. with water and read at 650 mμ.

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[27] F. Bertolani and A. Bergamini, *Giorn. biochim.* 2, 310-9 (1953).

2,5-DIHYDROXYBENZOIC ACID, GENTISIC ACID

The familiar diazo reaction with bilirubin is applicable to gentisic acid.[28] An alternative is oxidation by acid potassium ferricyanide.[29]

Procedure—*By diazotized sulfanilic acid. Serum.* As diazo reagent dissolve 1 gram of sulfanilic acid in 200 ml. of 1:20 hydrochloric acid. Chill and mix 10 ml. with 10 ml. of chilled 0.5% sodium nitrite solution. Add 2.5 ml. of concentrated ammonium hydroxide. Mix 0.5 ml. of bilirubin-free serum, 8.5 ml. of water, and 1 ml. of diazo reagent. Add a drop of 10% ferric chloride. Read at 540 mμ after 10 minutes.

By ferricyanide oxidation. Blood. Add 4 ml. of blood to 15 ml. of 95% ethanol and mix. Centrifuge after 1 hour. Decant and wash the residue with 5 ml. and 5 ml. of 95% ethanol. To the combined extracts add 50 ml. of 1:175 sulfuric acid and extract with 25 ml. of ether. Extract with four 15-ml. portions of ether. Evaporate the combined ether extracts to 50 ml. Extract the gentisic acid from the ether with 15 ml. of 0.3% sodium bicarbonate solution followed by three 10-ml. portions. Add 0.5 gram of kaolin to the alkaline extracts. Filter after 10 minutes and wash the filter with 0.3% sodium bicarbonate solution. Evaporate ethanol and ether from the alkaline extract and dilute to a known volume.

As a reagent mix 2 ml. of 3.3% potassium ferricyanide solution in 1:20 hydrochloric acid, 2 ml. of 9.5% solution of potash alum in 1:20 hydrochloric acid, and 96 ml. of 1:20 hydrochloric acid. To develop mix 5 ml. of sample with 2 ml. of this reagent and read.

o-AMINOBENZOIC ACID, ANTHRANILIC ACID

A reagent for free anthranilic acid, extracted from tissue samples with ether, is 2-(diethylaminoethyl)-1-naphthylamine oxalate.[30] Glucuronated anthranilic acid is also determined after hydrolysis to the free acid by sodium hydroxide. Aniline, acetanilide, 5-hydroxyanthranilic, and *N*-acetylanthranilic acids do not interfere.

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[28] B. A. Mendioroz, *Arch. soc. biol. Montevideo* **19**, 97-100 (1952).

[29] M. Rossanda, *Atti soc. lombarda sci. med. biol.* **5**, 74-7 (1949-50).

[30] Y. Shirai and T. Ohkubo, *J. Biochem.* (*Japan*) **41**, 337-40 (1954).

Procedure—*Tissue.* Add a 200-mg. sample to 4 ml. of Krebs-Henseleit phosphate Ringer solution in which the magnesium sulfate has been replaced by magnesium chloride. Aerate with oxygen for 1 minute and incubate at 37.5° for 1.5 hours. Remove the tissue sample and cool the solution. Add 6 ml. of water and 2 ml. of 10% trichloroacetic acid. Filter after 15 minutes. Acidify an 8-ml. aliquot of the filtrate using Congo red as the indicator. Extract free anthranilic acid 3 times with 15-ml. portions of ether. The glucuronated anthranilic acid remains in the aqueous layer. Re-extract the free acid from the ether with two 8-ml. portions of 0.4% sodium hydroxide solution. This represents the free anthranilic acid.

Hydrolyze the aqueous layer containing glucuronated anthranilic acid by heating with 2 ml. of 4% sodium hydroxide solution at 55° for 1 hour. Neutralize and extract the liberated acid 3 times with 15-ml. portions of ether and re-extract from the ether with two 8-ml. portions of 0.4% sodium hydroxide solution. This represents the glucuronated anthranilic acid.

To 10 ml. of each extract containing anthranilic acid in 0.4% sodium hydroxide solution, add 0.5 ml. of 1:2 hydrochloric acid, and 1 ml. of 0.1% sodium nitrite solution. After a few minutes add 1 ml. of 0.5% ammonium sulfamate solution and 1 ml. of 0.1% 2-(diethylaminoethyl)-1-naphthylamine oxalate solution and heat at 37° for 3 hours. Read at 550 mμ.

Cadmium Anthranilate

This is an anthalmintic used for swine. It is extracted from the feed by hydrochloric acid. Then the determination is of the anthranilate radical by diazotizing and coupling with *N*-(1-naphthyl)-ethylenediamine dihydrochloride.[31]

Procedure—Treat 2 grams of feed with 1:35 hydrochloric acid and dilute to 100 ml. with that acid. Shake intermittantly over a 30-minute period and let settle. Filter with suction on a Filter-Cel mat in a Büchner funnel. Discard the first part of the filtrate so obtained.

Mix a 5-ml. aliquot containing up to 0.1 mg. of anthranilic acid with 5 ml. of 95% ethanol, 1 ml. of 1:1 hydrochloric acid, and 1 ml. of fresh 0.1% sodium nitrite solution. After 5 minutes add 1 ml. of 0.5% solution of ammonium sulfamate. After 2 minutes add 1 ml. of 0.1% solution of

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[31] H. F. Beckman and L. S. Feldman, *J. Agr. Food Chem.* 7, 350-1 (1959).

N-(1-naphthyl)-ethylenediamine dihydrochloride. Heat at 65° for 30 minutes and cool. Read at 538 mμ against a reagent blank.

p-Aminobenzoic Acid

The method is applied to determination of *p*-aminobenzoic acid occurring in procaine solutions due to decomposition of the latter. The procaine is extracted by chloroform and the test substance determined by diazotizing and coupling.[32]

Procedure—Make 10 ml. of procaine solution alkaline with about 1 gram of disodium hydrogen phosphate. Extract with 10 ml. and 10 ml. of chloroform and discard the extracts. Chill 5 ml. of the aqueous solution and add 1 ml. of 1:5 hydrochloric acid. Add 0.5 ml. of chilled 0.5% sodium nitrite solution. Mix and add 0.5 ml. of 5% thymol solution in 8% sodium hydroxide solution. Mix, add 1 ml. of 40% sodium hydroxide solution, and read against a reagent blank.

p-Aminosalicylic Acid

Para-aminosalicylic acid reacts with vanillin with the formation of an azomethine, *N*-(3-methoxy-4-hydroxy-benzylidene)-*p*-aminosalicylic acid.[33] This is a water-soluble yellow compound with a maximum absorbance at 410 mμ. Isonicotinic acid hydrazide interferes. Trichloroacetic acid is preferred to tungstic acid as a protein precipitant becaus the latter may precipitate and interfere. Streptomycin, dihydrostreptomycin, and viomycin do not interfere. Beer's law is followed for 0.0001-0.0015%. A diazo reagent prepared from *p*-sulfanilic acid is also applicable.[34] An alternative diazo reagent from *N*-1-naphthylethylenediamine gives a purple color.[35] In one form the *p*-aminosalicylic acid is decarboxylated in acid solution, then diazotized and coupled with added *p*-aminosalicylic acid.[36]

Sodium nitropentacyanocobaltate (II), $Na_4Co(CN)_5NO_2$, reacts with aromatic compounds with amino and hydroxyl groups in the meta position to give an orange color, which in the case of *p*-aminosalicylic acid is read at 440 mμ.[37] The color reaches a maximum intensity after 1 hour and conforms to Beer's law up to 0.075 mg. per ml. A similar reaction is given by *m*-aminophenol. The reagent is unstable and must be prepared for each determination. Either *p*-aminosalicylic acid or its sodium salt are read at 265 mμ.[38]

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[32] J. Richter, *Arzneimittel-Forsch.* 4, 686-7 (1954); *Ibid.* 7, 200-1 (1957).

[33] E. N. Deeb and G. R. Vitagliano, *J. Am. Pharm. Assoc.* 44, 182-5 (1955).

[34] I. Kidani, T. Nakashima, Y. Kochi, and S. Kasahara *Bull. Natl. Hyg. Lab (Tokyo)* No. 72, 91-3 (1954); V. Temmere and K. Segliņš, *Trudy Inst. Ekspt. Med. Akad. Nauk Latv. S.S.R.* No. 15, *Voprosy Tuberkuleza* No. 2, 57-68 (1957).

[35] E. K. Marshall, Jr., *Proc. Soc. Exptl. Biol. Med.* 68, 471-2 (1948); J. Wagner *Rozhledy Tuberk.* 16, 207-10 (1956).

[36] Maurice Pesez, *Bull. soc. chim. biol.* 31, 1369-72 (1949); Vincente de Paul Castillio, *Rev. quím. e farm. Rio de Janeiro* 16, No. 10, 271-9 (1951).

Procedure—*By vanillin. Plasma.* Dilute a 2-ml. sample to 12 ml. with water. Mix with swirling and add 4 ml. of 25% trichloroacetic acid. Stopper and shake vigorously. Centrifuge for 5 minutes and filter. As a reagent, dissolve 10 grams of vanillin in 50 ml. of 95% ethanol and dilute to 100 ml. with water. To 8 ml. of the protein-free centrifugate containing 0.02-0.3 mg. of *p*-aminosalicylic acid, add 1 ml. of vanillin reagent and 1 ml. of 1:6 sulfuric acid. Mix by swirling and let stand for 10 minutes. Read at 410 mμ against a reagent blank.

By diazotized sulfanilic acid. Prepare a diazo reagent by mixing 3 ml. of chilled 1% *p*-sulfanilic acid solution in 1:9 hydrochloric acid with 3 ml. of chilled 1% sodium nitrite solution and 44 ml. of cold water. After 10 minutes add 2 ml. of 1% urea solution to remove interfering excess nitrite.

To a 2-ml. sample containing 0.02-0.04 mg. of *p*-aminosalicylic acid, add 0.5 ml. of 36% formaldehyde solution, 0.1 ml. of 1:10 hydrochloric acid, and 3 ml. of fresh diazo reagent. Heat for 30 minutes at 40°. Cool to room temperature, add 10 ml. of 4% sodium hydroxide solution, and read within 30 minutes at 494 mμ.

By p*-aminosalicylic acid. Blood.* Mix 2 ml. of blood with 6 ml. of water. After laking for 5 minutes add 2 ml. of 50% trichloroacetic acid solution. Heat for 30 minutes at 100° to decarboxylate the *p*-aminosalicylic acid to *m*-aminophenol. Centrifuge and decant. Cool in ice and add 1 ml. of 1% *p*-aminosalicylic acid as coupling agent. Add 1 ml. of chilled 2% sodium nitrite solution. Excess of added *p*-aminosalicylic acid is converted to 2,4-dihydroxybenzoic acid which does not interfere. Read the yellow photometrically.

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[37] P. A. Caccia, *Am. Rev. Tuberc. Pulmonary Diseases* 76, 1071-8 (1957); *Anal. Chem.* 31, 1306-8 (1959).

[38] Antonio Munoz Armestar, *Congr. farm. bioquim. peruano y convención farm. Norte, Actas y trabajos* 1953, 241-4; Cf. E. Ota, S. Nakajima, and S. Nakagami, *Ann. Rept. G. Tanabe Co. Ltd.* 1, 63-7 (1956).

Urine. Acidify 2 ml. with 0.5 ml. of 50% trichloroacetic acid. Complete as for blood from "Heat for 30 minutes. . . ."

By sodium nitropentacyanocobaltate (II). To prepare sodium nitropentacyanocobaltate (IV), slowly add a solution of 120 grams of sodium nitrite in 180 ml. of water to 30 grams of cobalt nitrate hexahydrate in 50 ml. of water and 15 ml. of glacial acetic acid. Add 18 grams of sodium cyanide and heat to 60° while mixing. Cool until the sodium nitropentacyanocobaltate (IV) crystals form. To prepare the sodium nitropentacyanocobaltate (II) reagent, mix equal volumes of 4% sodium nitropentacyanocobaltate (IV) solution and 16% sodium hydroxide solution.

Dilute a sample containing 0.005-0.125 mg. of sodium *p*-aminosalicylate to 1 ml. with water. Add 1 ml. of freshly prepared reagent and 1 ml. of 1:6 acetic acid to bring the pH to approximately 5. Any mineral acid may be substituted. Let stand for 1 hour at room temperature and add 7 ml. of water. Mix and read at 440 mμ against a reagent blank.

N-Benzoyl-*p*-aminosalicylic Acid

Similar to the determination of salicylic acid, *N*-benzoyl-*p*-aminosalicylic acid is estimated by its reaction with ferric ion to form a violet color.[39] *p*-Aminosalicylic acid and *m*-aminophenol do not interfere. If they are present they can be isolated by paper chromatography for determination. A satisfactory system is pyridine, butanol, saturated aqueous sodium chloride solution, 1:1 ammonium hydroxide at 6:6:5:3.

Procedure—Shake 0.17 gram of finely powdered tablets for 30 minutes with 100 ml. of 65% ethanol, dilute to 500 ml. with water, and filter. To a 4-ml. aliquot of the extract, add 15 ml. of water, and 2 ml. of 1% ferric nitrate solution in 1:99 nitric acid. Dilute to 25 ml. with water and read at 530 mμ.

Benemid, *p*-(di-*n*-propyl-sulfamyl)-Benzoic Acid

Benemid is extracted into chloroform from an acidic aqueous medium, re-extracted into dilute sodium hydroxide, and read in the ultraviolet. An alternate method involves shaking the chloroform extract with methylene blue to form a colored salt. The ultraviolet method has a greater accuracy except in the presence of interfering compounds as indicated.[40]

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[39] J. Vacek and M. Lastovkova, *Ceskoslov, farm.* 5, 75-7 (1956).

p-Aminohippuric acid, uric acid, ascorbic acid, thiamine, penicillin, aureomycin, streptomycin, and terramycin do not interfere with either method. In the methylene blue method there is some interference by 0.1 mg. of benzoic acid per 100 ml. but no interference from 0.05 mg. per 100 ml. Both concentrations interfere with the ultraviolet determination. *p*-Aminobenzoic acid interferes in concentrations of 0.04 mg. per 100 ml. with the ultraviolet method but not with the methylene blue method. There is no interference by 0.02 mg. per 100 ml. in either method. At 0.1 mg. per 100 ml. of hippuric acid there is interference with methylene blue, none at 0.05 mg. Either concentration prevents determination by the ultraviolet method. Concentrations of 0.002 mg. per 100 ml. of salicylic acid produce some interference in the methylene blue method and greater interference in the ultraviolet procedure, while 0.001 mg. per 100 ml. only interferes with the ultraviolet reading. Up to 0.04 mg. per 100 ml. of *p*-aminosalicyclic acid may be present in the methylene blue method while the level of interference with the ultraviolet method is 0.01 mg. per 100 ml. Quantities as small as 0.001 mg. per 100 ml. of acetylsalicylic acid cause some interference with the ultraviolet method while up to 0.05 mg. per 100 ml. can be tolerated in the methylene blue procedure. At the level of 0.05 mg. of sulfadiazine there is some interference in the methylene blue method while as little as 0.005 mg. per 100 ml. interferes in the ultraviolet.

There is no interference by sulfamerazine in the methylene blue procedure while over 0.025 mg. per 100 ml. interferes with the ultraviolet method. 5-Ethyl-5-(methyl-1-butenyl)-barbiturate, *N'*-methylcyclyhexenyl-methyl barbituric acid, and ethyl-(-1-methylbutyl)-thiobarbituric acid can be present in the determination with methylene blue, but 0.05 mg. per 100 ml. of any one of them interferes with the ultraviolet method. Beer's law is followed.

Procedure—*Directly in the ultraviolet.* Purify chloroform by shaking successively with 1 ml. of 4% sodium hydroxide solution and 1 ml. of 1:10 hydrochloric acid followed by several washings with water. Add 30 ml. of chloroform to a 1-ml. urine or plasma sample containing 0.02-0.09 mg. of benemid, and add 1 ml. of 1:10 hydrochloric acid. Shake 15 minutes, centrifuge for 3-5 minutes, and remove the aqueous layer. Shake

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[40] E. K. Tillson, G. S. Schuchardt, J. K. Fishman, and K. H. Beyer, *J. Pharmacol. Exptl. Therap.* **111,** 385-94 (1954)

20 ml. of the chloroform with 5 ml. of 0.4% sodium hydroxide solution for 5 minutes. Decant and centrifuge at low speed for 3 minutes. If the plasma contains a large amount of fat, centrifuge longer. Read at 242.5 mμ. The reagent blank using water in place of the sample should not have an optical density greater than 0.05.

By methylene blue. Prepare a phosphate buffer at pH 7 by mixing 2.7% monobasic potassium phosphate solution with 0.8% sodium hydroxide solution until the pH is reached. Shake a 1-ml. sample containing 0.02-0.09 mg. of benemid with 30 ml. of purified chloroform and 1 ml. of 1:10 hydrochloric acid for 15 minutes. Centrifuge for 3-5 minutes. To a 15-ml. aliquot of the chloroform extract add 15 ml. of 0.0125% methylene blue solution in pH 7 phosphate buffer and shake for 10 minutes. Centrifuge for 1 minute to separate the layers. Remove the aqueous phase and read the solvent layer immediately at 635 mμ against a reagent blank.

TETRAHYDROXYCYCLOHEXANE CARBOXYLIC ACID, QUINIC ACID

The reaction product of quinic acid with sodium nitroprussidepiperazine reagent is suitable for reading at 390 mμ.[41]

Procedure—Mix 1 ml. of 6.4% sodium periodate solution with a 1-ml. sample containing 0.025-0.12 mg. of quinic acid and let stand for 15 minutes at 30°. Add 1 ml. of 30% ethylene glycol solution. Shake well and let stand for 5 minutes at 30°. Add 1 ml. of carbonate buffer for pH 10.3 and 1 ml. of 0.05% sodium nitroprusside—0.05% piperazine solution. Heat for 5 minutes at 100° and cool for 5 minutes. Add 5 ml. of 95% ethanol, shake well, and read at 390 mμ.

PHTHALIC ACIDS

Ultraviolet spectrophotometry remains the prominent method for determination of phthalic acid (Vol. III, pp. 228-30). In the absence of maleic and fumaric acids it is applied to the saponification products of alkyd resins[42] and to lacquers.[43]

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[41] Masatoshi Nagasawa, *Bull. Agr. Chem. Soc. Japan* 22, 205-7 (1958).

[42] "1955 Book of ASTM Standards," Part 4, Method D-1307-54T, pp. 360-4, American Society for Testing Materials, Philadelphia, Pennsylvania.

[43] M. H. Swann, M. L. Adams, and G. G. Esposito, *Anal. Chem.* 27, 1426-9 (1955)

In recent years the isomers of phthalic acid have developed major industrial interest. Since the isomers show different points of maximum absorption at the longer wave lengths, they are read individually at 275, 281, and 287 mμ, the points at which the spread between the curves is greatest.[44] Each requires correction for the others. Long wave lengths are preferred to short wave lengths, in which meta and para isomers show strong absorption, to eliminate interference of water-soluble compounds. Accuracy to ±1% is obtained with the ortho and para isomers but for the meta isomer it is about ±1.5%.

Phthalic acid in phthalic anhydride is separated by dissolving away the anhydride with chloroform. The phthalic acid is read directly at 276 mμ.[45] In reading of phthalic anhydride at 291 or 300 mμ the acids do not absorb. The precision of the method is ±0.5%.

In analysis for phthalic acid in naphthalene oxidation products maleic acid should not be present at over 0.1 mg. per ml., and in the final dilution it should not exceed 0.004 mg. per ml.[46] Both the phthalic acid and maleic acid are separated from the interference of 1,4-naphthoquinone by precipitation with barium ion in ethanol followed by solution in hydrochloric acid. Excess barium ions are provided by addition of barium chloride. The reaction mixture is neutralized to a phenolphthalein end point with sodium hydroxide prior to addition of barium ion to prevent formation of polymerization products. Such polymers also dissolve in hydrochloric acid. The final reading is at 274 mμ.

A condensation reaction between *o*-phthalic acid or anhydride and hydroquinone produces a highly colored quinizarin compound suitable for determining *o*-phthalates in all types of coating materials. The yellow color is read in benzene or a violet color is read in aqueous alkali.[47]

Although both maleic acid and succinic acid form colored products with hydroquinone, neither interferes, since the color formed by maleic acid is not extracted into benzene and the color formed by succinic acid decomposes after 2 hours at 145°. Succinic acid is determined by this method in the absence of phthalic acid or by reading before and after the color fades. The adduct of maleic acid with isoprene forms a product with the reagent of the same color as with the phthalate. The adduct with butadiene does not develop a color. Phthalic ester plasticizers such dibutyl phthalate cannot be distinguished from polymeric phthalates by this method unless separated by charcoal sorption. This method is not as accurate as reading in the ultraviolet, but has the advantage of a fewer number of interferences.

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[44] M. H. Swann, M. L. Adams, and D. J. Weil, *Ibid.* 27, 1604-6 (1955).

[45] M. M. Agarwal and F. Spagnolo, *Ibid.* 25, 1412-3 (1953).

[46] W. P. Fassinger and C. E. Gonter, *Ibid.* 31, 1324-6 (1959).

[47] M. H. Swann, *Ibid.* 29, 1352-3 (1957).

Diisonitrosoacetone gives a magenta color with many materials including phthalic acid, phthalic anhydride, and diethylphalate.[48] Interfering substances are diisopropyl phosphonofluoridate and isopropyl methylphosphonofluoridate, tetraethylpyrophosphate, methylphosphonodichloridate, phosphorus oxychloride, *N*-bromosuccinimide, benzenesulfonylchloride, benzoylchloride, paraoxan, parathion, and tabun.

Traces of acids, presumably terephthalic acid, in dimethylterephthalate are determined by the reduction of color on reaction with the potassium salt of bromothymol blue in toluenedimethylformamide solution.[49] Water and carbon dioxide must be rigorously excluded.

Procedure—*Phthalic acid and phthalic anhydride. Direct reading.*[50] Disperse a 1-gram sample in 20 ml. of chloroform. Filter with suction through fritted glass and wash with three 20-ml. portions of chloroform. Wash the lower rim of the filter with chloroform and add to the filtrate. To determine phthalic anhydride dilute the chloroform extract to 250 ml. with chloroform and read at 291 mμ or 300 mμ against chloroform.

To the residue, add 10 ml. of boiling 0.4% sodium hydroxide solution and stir. Filter and wash with two 10-ml. portions of the sodium hydroxide solution. Neutralize the combined filtrates to pH 2 with 1:3 hydrochloric acid and dilute to 100 ml. with 1:100 hydrochloric acid. Read at 276 mμ against 1:100 hydrochloric acid.

Mixed phthalic acids.[51] *Direct reading.* Reflux a sample containing a total of 50 mg. of mixed acids with 50 ml. of absolute ethanol until dissolved. Add 10 ml. of dry benzene and 3 ml. of 11.2% potassium hydroxide solution in absolute ethanol. Reflux for 1 hour and add 150 ml. of dry benzene. Stopper and cool under water. Filter on a Gooch crucible and wash with benzene followed by a washing with ether. Dry at 105°, dissolve in water, and dilute to 100 ml. Dilute a 10-ml. aliquot to nearly 100 ml. with 50% methanol. Add 2 ml. of 1:1 hydrochloric acid and make up to volume with 50% methanol. Read at 275, 281, and 287 mμ. Calculate the isomers as follows:

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[48] S. Sass, W. D. Ludemann, B. Witten, V. Fischer, A. J. Sisti, and J. I. Miller, *Ibid.* **29,** 1346-9 (1957).

[49] A L. Hensley, *Ibid.* **32,** 542-3 (1960).

[50] M. M. Agarwal and F. Spagnolo, *Ibid.* **25,** 1412-3 (1953).

[51] M. H. Swann, M. L. Adams, and D. J. Weil, *Ibid.* **27,** 1604-6 (1955).

(*m*) isophthalic acid $= -0.975A_{275} + 1.254A_{281} - 0.3223A_{287}$

(*o*) orthophthalic acid $= 0.383A_{275} - 0.170A_{281} - 0.165A_{287}$

(*p*) terrephthalic acid $= 0.283A_{275} - 0.493A_{281} + 0.310A_{287}$.

Naphthalene oxidation products.[52] Dilute a sample containing 10-15 mg. of phthalic acid to 20 ml. with water. Add a few drops of phenolphthalein indicator solution and dropwise add 0.4% sodium hydroxide solution until a faint pink color is visible. Add 10 ml. of 0.208% barium chloride solution in carbonate-free water followed by 100 ml. of absolute ethanol. Stir vigorously and let stand for 1 hour to ensure complete precipitation of the phthalates. Filter with suction, using absolute ethanol to transfer and wash the precipitate. Dissolve the precipitate in warm 1:99 hydrochloric acid and dilute to 250 ml. with the hydrochloric acid. Read at 274 mμ against hydrochloric acid.

Phthalic acid, phthalic anhydride, and diethyl phthalate by diisonitrosoacetone.[53] As a buffer for pH 8.4 mix 17 ml. of 2% sodium hydroxide solution with 500 ml. of 0.62% boric acid solution. Mix 1 ml. of aqueous solution containing 0.005-0.06 mg. of the test substances with an equal volume of 0.4% aqueous solution of diisonitrosoacetone. Add 3 ml. of the buffer and let stand for 7 minutes. Read at 486 mμ or 580 mμ.

Alkyd resins. Maleic and fumaric acids absent. Direct reading.[54] Weigh a sample to yield 0.8-1.2 grams of potassium alcohol phthalate upon saponification. Dissolve in 150 ml. of benzene, warming if necessary, and add 60 ml. of 60% potassium hydroxide solution in absolute ethanol which has stood overnight protected against carbon dioxide absorption. Warm at 40° for 1 hour, gradually raise the temperature until the alcoholic solution boils gently, and reflux for 90 minutes. Wash down the condenser with a few ml. of a 1:3 mixture of anhydrous ethanol and benzene. Remove the condenser, stopper, and cool rapidly in ice. Filter immediately, using the alcohol-benzene solution for transferring and washing. Continue washings until the washings are no longer alkaline to phenophthalein. Approximately 75 ml. of wash solution is required. Do not allow air to be drawn through the precipitate since it is hygroscopic.

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[52] W. P. Fassinger and C. E. Gonter, *Ibid.* **31**, 1324-6 (1959).

[53] S. Sass, W. D. Ludemann, B. Witten, V. Fischer, A. J. Sisti, and J. I. Miller, *Ibid.* **29**, 1346-9 (1957).

[54] "1955 Book of ASTM Standards," Part 4, pp. 360-4, American Society for Testing Materials, Philadelphia, Pennsylvania.

Add 25 ml. of ether and filter with suction. Heat the crucible at 60° for 1 hour, cool to room temperature in a desiccator, and weigh the alcoholate of the phthalic acid. Extract by passing 200 ml. of water by suction through the precipitate. Add 20 ml. of concentrated hydrochloric acid. A precipitate or cloudiness will appear in the presence of sebacic acid or other slightly soluble acidic material. If clear, dilute to 2 liters with water. If not, filter with suction, wash with 100 ml. of water, and dilute the filtrate and washings to 2 liters with water. Dilute a 50-ml. aliquot to 250 ml. with 1:100 hydrochloric acid. Read at 276 mμ against 1:100 hydrochloric acid.

Maleic and fumaric acids present. Prepare an absorption curve fo these acids. Calculate from these simultaneous equations:

$$A_2 = a_p C_p + a_i C_i$$

$$B/2f = F_p C_p + F_i C_i$$

A_2 = absorbance of final solution
a_p = absorbance of pure phthalic acid
a_i = absorbance of the interfering acid
C_i = measured concentration of interfering acid
B = grams of dried precipitate
f = dilution factor of final solution.

F_p and F_i = factors for conversion of phthalic and interfering acids t their potassium salts as present in the precipitate. F_p for phthalic acid = 1.735. F_p for maleic or fumaric acid = 1.654. This assumes that they ar not in the precipitate as alcoholates.

As another technic for alkyd resins, dissolve a sample containing 0.1-0.5 gram of total phthalic acid in 10 ml. of benzene. Add 100 ml. of 2.8% potassium hydroxide solution in ethanol and agitate at room temperatur until precipitation begins. Warm at 45° for at least 4 hours and reflu using an air condenser for 1 hour. Add 150 ml. of dry benzene, stopper cool and let stand for 30 minutes.

Complete as for mixed phthalic acids from "Filter on a Gooch crucible. . . ."

Alkyd lacquer. Direct reading.[55] Weigh a clear sample estimated t contain 0.1 gram of phthalic acid. Pigmented samples must be centrifuged to obtain a clear vehicle, thinning prior to centrifuging if necessary

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[55] M. H. Swann, M. L. Adams, and G. G. Esposito, *Anal. Chem.* 27, 1426-9 (1955)

Dissolve in 50 ml. of methyl isobutyl ketone and 200 ml. of 0.7% potassium hydroxide solution in absolute ethanol. Reflux for 3 hours and filter through asbestos while warm. Wash with absolute ethanol, dry at 105°, and cool. Dissolve the dry precipitate in water, acidify with 10 ml. of concentrated hydrochloric acid, and dilute to 1 liter. After standing for 18 hours, filter and discard the first 25-30 ml. of filtrate. Dilute a 50-ml. aliquot of the filtrate to 100 ml. with 1:110 hydrochloric acid.

Read at 276 mμ against 1:110 hydrochloric acid. In the presence of known amounts of maleic and fumaric acids, measure the absorbance of a pure sample of the interfering acids at 276 mμ in 1:100 hydrochloric acid and apply the necessary correction. Calculate the concentration of phthalic acid in grams per liter as follows:

$$Cp = A/a_p$$

where Cp = concentration of phthalic acid in grams per liter

A = measured absorbance

a_p = absorptivity value of pure phthalic acid.

Nitrocellulose lacquer. Direct reading. Weigh a sample containing 0.3 gram of lacquer-grade nitrocellulose. Saponify, following the procedure under "Alkyd lacquer" starting at "Dissolve in 50 ml. of methyl isobutyl ketone . . ." and ending with ". . . dry at 105° and cool." Dissolve the residue in water, add 10 ml. of concentrated hydrochloric acid, and dilute to 1 liter with water. Let stand at least 18 hours and filter, discarding the first 25-30 ml. of filtrate. Read at 270 and 276 mμ.

The absorbance should fall within the range of 0.25 to 0.50. If the readings are outside this range, the dilution factor should be adjusted but anything greater than a fourfold final dilution should be avoided. Calculate phthalic acid as follows:

$$C_p = \frac{a_{270n}A_{276} - a_{276n}A_{270}}{a_{270n}a_{276p} - a_{276n}a_{270p}}$$

in which C_p = concentration in grams per liter of phthalic acid in the final diluted solution

a_{270p} and a_{276p} = absorptivities of phthalic acid at 270 and 276 mμ respectively

a_{270n} and a_{276n} = absorptivities of the nitrocellulose saponification product at 270 and 276 mμ respectively as determined above

A_{270} and A_{276} = the average absorbances at 270 and 276 mμ respectively.

Coating materials, dried or liquid, colored or colorless.[56] Dissolve a sample containing 0.1-0.3 gram of nonvolatile matter in benzene or acetone and dilute to 100 ml. Dry an aliquot of 5-ml. or less containing not more than 4 mg. of phthalic anhydride at 105°. Add exactly 1 gram of hydroquinone and 2 ml. of concentrated sulfuric acid. Heat at 105-110° for 30 minutes and at 145° for 2 hours, agitating gently occasionally. Heating over 145° causes foaming of the reagent and decomposition.

Cool, slowly dilute with water to 25-30 ml., and add 75 ml. of benzene. Shake, allow the layers to separate, and re-extract the aqueous layer with 20 ml. of benzene. Wash each benzene extract separately with water and combine. Filter and dilute to 100 ml. with benzene. Read at 480 mμ.

Acid in dimethyl terephthalate. As reagent dissolve 0.4 gram of bromothymol blue in 400 ml. of moisture- and carbon dioxide-free dimethylformamide. Add 2.8% potassium hydroxide in absolute ethanol until a permanent dark blue is obtained. Dilute to 500 ml. with dimethylformamide and protect at all times with an Ascarite tube.

Dissolve a sample equivalent to 0.05-0.2 mg. of potassium hydroxide in about 75 ml. of 1:3 toluenedimethylformamide. Add 2 ml. of indicator solution and dilute to 100 ml. with the mixed solvent. Read within 1 hour at 610 mμ against a solvent blank. The calibration curve must have been prepared with the same batch of reagent.

Diethyl, Dibutyl, and Dioctyl Phthalates

Diethyl, dibutyl, and dioctyl phthalates are developed as hydroxami acids and read as the ferric salt.[57] The method is not applicable to dimethyl or diphenyl phthalates. Maximum absorbance for the ferric hydroxamate colors of diethyl, dibutyl, and dioctyl phthalates occurs a 540 mμ. But the color is read at 650 mμ to eliminate interference o 2-nitrodiphenylamine. Interference of nitroglycerin and dinitrotoluene i removed by reduction with titanous chloride. Lead stearate, triacetin and up to 2 ml. of petroleum ether can be tolerated. Beer's law is followed

Procedure—*Ball propellants.* Reflux a 0.1-0.3 gram sample containing 6-12 mg. of diethyl or dibutyl phthalate or 10-18 mg. of diocty phthalate with 20 ml. of anhydrous ethyl ether for 1 hour. Evaporate th ether by placing the flask in water at 60°. Add 10 ml. of glacial aceti acid and cool under water for 30 seconds. Add 10 ml. of 50% sodiu acetate solution and 10 ml. of 20% titanous chloride solution to reduce nitroglycerin and dinitrotoluene. Stopper, swirl for 1 minute, and let stand for 2 minutes. Wash the stoppers with water and dilute to 200 ml. with water.

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[56] M. H. Swann, *Anal. Chem.* **29,** 1352-3 (1957).

[57] George Norwitz, *Ibid.* **31,** 2012-5 (1959).

Extract the phthalate with 85 ml. of petroleum ether followed by two 50-ml. portions, shaking for 1 minute for each extraction. Shake the combined petroleum ether extracts with 25 ml. of 5% sodium carbonate solution to eliminate interference from acetic acid extracted with the petroleum ether. Evaporate the petroleum ether by placing the flask in water at 100°.

To prepare the ferric perchlorate reagent, dissolve 1.60 grams of pure iron by warming gently with 20 ml. of 70% perchloric acid solution. Take up in 20 ml. of water, cool to room temperature under water, and dilute to 200 ml. with 95% ethanol. To a 40-ml. aliquot, add 12 ml. of 70% perchloric acid. Cool to room temperature with water and dilute to 1 liter with 95% ethanol.

To prepare the hydroxylamine-sodium hydroxide reagent,[58] reflux 12.5 grams of hydroxylamine hydrochloride with 100 ml. of methanol for a few minutes. Reflux 12.5 grams of sodium hydroxide with 100 ml. of methanol for a few minutes. Mix equal volumes of the two solution and filter off the precipitated sodium chloride.

To the sample add 5 ml. of 95% ethanol and 3 ml. of hydroxylamine-sodium hydroxide reagent. Prepare a blank by adding 3 ml. of hydroxylamine-sodium hydroxide reagent to 5 ml. of 95% ethanol. Cover both and heat at 65-67° for 30 minutes in an oven. Cool immediately under water for 1 minute and in ice and water for 7 or more minutes. Add 30 ml. of ferric perchlorate reagent to the blank, dilute to 250 ml. with reagent, and set the spectrophotometer to 100% transmittance at 650 mμ with the blank. Make similar additions to the sample and read.

DIOCTYL PHTHALATE AND 2-NITRODIPHENYLAMINE

Dioctyl phthalate in ethanol solution has an absorption maximum at 276 mμ.[59] In samples containing nitroglycerin, the nitrate groups are reduced to free nitrogen by the catalytic hydrogenolysis with platinum oxide or palladium black. Samples containing 2-nitrodiphenylamine are treated in two ways depending on whether a determination of 2-nitrodiphenylamine is desired. In samples where the amine is also to be measured, the reduced sample is passed through a cation-exchange resin to remove the reduction products of the amine and the phthalate is read at 276 mμ while the amine is read in an unreduced portion of the sample at 425 mμ. In samples where the amine is not being determined, the phthalate is precipitated as the dipotassium salt, acidified with hydrochloric acid, and read. Beer's law is followed for 0.016-0.024 mg. of potassium acid phthalate.

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[58] R. F. Goddu, N. F. LeBlanc, and C. M. Wright, *Ibid.* 27, 1251-4 (1955).

[59] H. Stalcup, M. I. Fauth, J. O. Watts, and R. W. Williams, *Ibid.* 29, 1482-5 (1957)

Procedure—*Propellants. Dioctyl phthalate and 2-nitrodiphenylamine simultaneously.* Fill a glass column 3 cm. in diameter and at least 25 cm. long, fitted with 29/26 ground-glass joints, to a height of 20 cm. with Amberlite IR-120 resin. Wash the resin thoroughly with 95% ethanol at room temperature and finally with hot 95% ethanol. Measure the absorbance of the effluent and continue washing until the absorbance is negligible. Grind a 7.5-gram sample to 20-mesh and extract with 100 ml. of 95% ethanol for 6 hours. Cool, filter, and dilute to 250 ml. with 95% ethanol.

Transfer a 50-ml. aliquot to a pressure bottle, add 0.3 gram of palladium black. Wash down the walls of the bottle to prevent flashing. Flush 5 times with hydrogen, adjust the hydrogen pressure to 30 pounds per square inch, and continue the reduction of nitroglycerin and 2-nitrodiphenylamine for 1 hour. Release the pressure and filter into the cation-exchange column to remove reduction products of 2-nitrodiphenylamine. Wash the pressure bottle and filter paper with 95% ethanol and add the washings to the column. Dilute the effluent to 250 ml. with 95% ethanol. Run additional 95% ethanol through the column as a blank. Read at 276 mμ. To correct for the absorbance of the solvent cell and the solution cell calculate dioctyl phthalate as follows:

$$\%\ \text{dioctyl phthalate} = \frac{D \times 28.78 \times V}{\text{mg. of sample}}$$

in which

D = the absorbance

V = the final volume of the eluate in ml.

28.78 = the ratio of the concentration of the phthalate in mg. per 100 ml. to the absorbance.

Dioctyl phthalate alone. Extract a 1-gram 20-mesh sample with 65 ml. of a 2:1 mixture of pentane-methylene chloride for 4 hours at 100° Evaporate to dryness at 100° under a gentle stream of air. Transfer to a pressure bottle with several portions of anhydrous isopropanol totaling

40-50 ml. Add 0.2 gram of platinum oxide catalyst and wash down the catalyst adhering to the walls to prevent flashing. Flush 3 times with hydrogen, adjust the hydrogen pressure to 30 pounds per square inch, and allow the hydrogenolysis of nitroglycerin to continue with shaking for 20 minutes and for sample containing 2-nitrodiphenylamine until the orange color of the compound disappears. Release the pressure and filter with mild suction. Wash the residue and the bottle three times with 10-ml. portions of isopropanol. Avoid a rapid flow of air through the filter funnel after the solvent has been filtered because of possible fire hazard.

Dilute to 100 ml. with isopropanol and concentrate to 50 ml. at low heat. To remove residual ammonia which interferes with phthalate recovery and to precipitate the phthalate as the potassium salt, add 25 ml. of 2.8% potassium hydroxide solution in absolute isopropanol to the hot solution. Reflux gently for 20-25 minutes using a short air condenser. Take up in 25 ml. of benzene and filter with suction. Wash the precipitate with several portions of a 2:1 mixture of isopropanol and benzene and finally wash with isopropanol to remove benzene.

Dissolve the precipitate in water and acidify with 5 ml. of concentrated hydrochloric acid. Dilute to 250 ml. with water and mix thoroughly. Read at 276 mμ against 1:110 hydrochloric acid.

NAPHTHENIC ACIDS

Naphthenic acids in aqueous alkaline solutions are measured by the turbidity formed by acidification with mineral acid.[60] Solutions of sodium chloride or potassium chloride or 5% ethanol have no effect on the development and density of the turbidity. The light absorption is almost directly proportional to the concentration of the naphthenic acids.

Procedure—*Mineral oil.* Saponify a 100-gram sample by heating with 50 ml. of 5.6% potassium hydroxide solution at 60° for 10 minutes. Separate the aqueous layer and repeat the saponification with two 50-ml. portions of 5.6% potassium hydroxide solution. Dilute the aqueous portions with water, and acidify an aliquot with 1:10 hydrochloric acid to pH 1-2.5. Dilute to 100 ml. and measure the turbidity after 5 minutes. If more than 0.1% of naphthenic acids is present, carry out the saponification with 5.6% potassium hydroxide solution in 30-40% ethanol solution. Distil off the ethanol prior to acidification.

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[60] M. Nedorost and V. Simanek, *Chem. Listy* 49, 1188-92 (1955); *Práce Uslavu pro Naftový Výzkum* Ser. E, No. 17/21, 57-70 (1956).

SHIKIMIC ACID, 3,4,5-TRIHYDROXY-1-CYCLOHEXENE-1-CARBOXYLIC ACID

Shikimic acid, a precursor of aromatic amino acids in bacteria and fungi and a precursor of lignin in higher plants, is quantitatively oxidized to aconitic dialdehyde, 2-pentene-1, 5-dialdehyde-3-carboxylic acid which condenses with aniline to form a polymethene pigment.[61] Sodium periodate is the oxidizing agent. Citric, malic, and oxalic acids may be present in concentrations 3 times that of shikimic acid. Tartaric, succinic, fumaric, malonic, and quinic acids do not react. Sugars and amino acids do not react under the conditions of the procedure. However, polyhydroxy compounds present in large quantities react with periodate and are therefore removed by ion exchange in a strong-base anion-exchange resin in the carbonate form. Aromatic hydroxy acids form a brown color with periodate thereby requiring a correction involving a control test with water in place of aniline or an extraction prior to determination with ether.

Procedure—*Plant material.* Crush a 10-gram sample and extract twice with 100-ml. portions of boiling methanol for 1 hour each. Distil off the solvent and dissolve the residue in 30 ml. of water. Discard the insoluble portion. Pack a glass tube 0.8 cm. in diameter with 0.5 gram of strong-base anion-exchange resin, Amberlite IRA 410. Change the resin to the carbonate form by treatment with 50 ml. of 6.2% sodium carbonate solution. Wash the resin with water until the effluent is no longer alkaline. Pass the plant extract through at a rate of 0.5-1 ml. per minute. The resin adsorbs the organic acids. Wash with water until the effluent is colorless. Treat with 30 ml. of 5.7% ammonium carbonate solution at the rate of 0.5-1 ml. per minute to elute organic acids. Remove the excess ammonium carbonate from the effluent by evaporation at 100° to approximately 5-6 ml. Dry in a vacuum desiccator and dissolve in 5 ml. of water.

Prepare a pH 4.7 acetate buffer by mixing equal volumes of 1:19 acetic acid and 8.2% sodium acetate solution. As an oxidizing agent dissolve 160.5 grams of sodium periodate in 25 ml. of this buffer. Prepare the aniline reagent by saturating water with freshly distilled aniline at 25°. To 1 ml. of the neutral solution containing 0.04-0.2 mg. of shikimic acid, add 1 ml. of periodate oxidizing agent. Heat at 30° for 15 minutes. Add 1 ml. of 1% ethylene glycol solution and heat at 30° for 15 minutes. Stir in 1 ml. of aniline reagent and let stand at room temperature for 5 minutes. Add 5 ml. of absolute ethanol, stir, and read the red color at 510 mμ.

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[61] S. Yeshida and M. Hasegawa, *Arch. Biochem. and Biophys.* **70**, 377-81 (1957).

RESIN ACIDS

Resin acids in rosin size are determined by extracting as the copper salt into petroleum ether.[62] Beer's law is followed at concentrations up to 20 grams per liter. The test is reproducible to ±2%.

Procedure—*Rosin size.* Shake 40 ml. of petroleum ether with a 0.5-1% solution of rosin size for 10 minutes. Add 10 ml. of 3% cupric acetate solution to 10 ml. of the petroleum ether extract and shake for 5 minutes. Read the petroleum ether layer.

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[62] E. Ya. Vinetskaya and A. A. Kupenskaya, *Bumazh. Prom.* **31,** No. **1, 10-13** (1956).

CHAPTER 17

COMPLEX ACIDS AND DERIVATIVES

Santonin (Cf. Vol. III, p. 444) and bisatin are developed, the latter after hydrolysis with 10% ethanolic sodium hydroxide, by oxidation with potassium ferricyanide.[1] They are read at 500 and 560 mμ respectively. Santonin is read directly at 244-6 mμ.[2]

Up to 40 mg. of santonin can be determined as its 2,4-dinitrophenylhydrazone. The color is stable for 1 hour.[3] For this, dilute a sample in a 1:1 mixture of 28% ammonium hydroxide and water with acetone so that the final solution contains 5.6 grams of ammonia per 100 ml. Then develop as the 2,4-dinitrophenylhydrazone.

Adenylic acid is read at 210 mμ.[4] Glycyrrhizinic acid is read directly at 248 [5] or 252 mμ.[6] Tannins are complex acids but are determined largely by their phenolic properties. Therefore they are given on page 137.

For determination of glucuronic acid lactone by thymol see page 197. For determination by aminodiphenyl see page 168. For determination by phenol in sulfuric acid see page 168. For determination of galacturonic acid by aminodiphenyl see page 168. For determination by phenol in sulfuric acid see page 167.

For determination of mannuronic acid lactone by phenol in sulfuric acid see page 167. For determination of furethrin, *dl*-2-(2-furfuryl)-4-hydroxy-3-methyl-2-cyclopenten-1-one ester of *dl*-cistranschrysanthemum monocarboxylic acid, by sodium sulfide, see page 433.

Uric Acid

A modification of the phosphotungstic acid method (Vol. III, pp. 434-8) for uric acid replaced sodium cyanide with glycerine-silicate to intensify the reaction and employs the phosphotungstic acid in a dual role of reagent and protein precipitant.[7] Sodium polyanetholsulfonate is added as a stabilizer and to prevent turbidity. Alkali added before protein precipitation contributes to the reproducibility of the modified method. In this modification there is no interference from ascorbic acid, ergothioneine, glutathione, or cystine.

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[1] Y. Tanase and S. Umeda, *Ann. Rept. Fac. Pharm. Tokushima Univ.* **4,** 1-5 (1955).

[2] Yoshisuke Natsume, *Folia Pharmacol. Japan* **48,** No. 1, Proc. 2 (1952).

[3] T. Omura, Y. Kimura, and H. Murayama, *J. Pharm. Soc. Japan* **76,** 743-4 (1956).

[4] M. V. Buell and R. E. Hansen, *Science* **126,** 842 (1957).

[5] V. M. van Katwijk and L. G. Huis in't Veld, *Rec. trav. chim.* **74,** 889-96 (1955).

[6] H. Hada and M. Inagaki, *Yakugaku Zasshi* **78,** 795-7 (1958).

In the standard procedure for uric acid with phosphotungstic acid, the maximum color is produced by a sodium cyanide concentration of 2.3-3.1%. Urea is added to stabilize the cyanide reagent. Beer's law is followed to 0.025 mg. with reagents of 14% and 19% concentration and to 0.01 mg. for 9% reagent concentration.

Changes in alkalinity of the reaction mixture influence the results.[8] The effect of amino acids can be diminished by diluting the test solutions. Addition of glacial acetic acid prevents cloudiness which forms with the reduction of the phosphotungstic reagent in the presence of sodium carbonate.[9]

In the determination of uric acid by arseno-18-tungstic acid (Vol. III, p. 438), a sodium silicate-glycerine solution replaces cyanide. Proteins are precipitated with sodium tungstate in an acid medium.[10] Turbidity does not interfere.

With the two reagents chemically combined in an arsenophosphotungstic acid reagent, pH is an important factor in color development.[11] The blue reaction mixture, when read at 890 mμ, has a pH of 8.1. At pH 5.4 the color is yellow and over pH 8.4 a white precipitation occurs.

To counteract the high rate of decomposition of the sodium cyanide reagent, urea is added as a stabilizer, or the reagent is allowed to stand 2 weeks before use to avoid the high initial rate of decomposition of a fresh reagent. With fresh cyanide solutions Beer's law is followed at concentrations above 0.04 mg. per 50 ml. of final solution. When the reagent is allowed to stand for 2 weeks, Beer's law is followed for lower concentrations. At 5-33° the absorbancy does not change while at 33-50° the absorbancy increases 9%. Disodium dihydrogen ethylenediamine tetraacetate, is used as a buffering and complexing agent. Allantoin, barbituric acid, guanine, hydantoin, urea, and xanthine do not interfere at concentrations of 0.1 mg. per 50 ml. of reaction mixture. Alloxan interferes at this concentration.

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[7] R. M. Archibald, *Clin. Chem.* 3, 102-5 (1957); C. Alper and J. Seitchik, *Ibid.* 3, 95-101 (1957).

[8] A. E. Steel, *Biochem. J.* 68, 306-9 (1958).

[9] P. Valori and A. Antonini, *Fegato,* 3, 463-70 (1957).

[10] E. R. Hausman, G. T. Lewis, and J. S. McAnally, *Clin. Chem.* 3, 657-8 (1957).

[11] D. R. Norton, M. A. Plunkett, and F. A. Richards, *Anal. Chem.* 26, 454-7 (1954).

The enzymatic method for uric acid (Vol. III, p. 435) depends on the decrease in optical density of the uric acid after addition of uricase.[12] Then the extinction difference at 290-295 mμ before and after adding uricase measures the uric acid in blood, urine, and spinal fluid. Deproteinizing is usually necessary. In one modification, trichloroacetic acid is added to stop the enzyme activity and the sample is read against a blank which has not been incubated with enzyme. In another modification the sample and enzyme are read before and after incubation. The enzymatic method is accurate and specific but requires exactly controlled conditions. New standards are required with each new batch of enzyme preparation.[13] Metabolites, urochrome, and indoxyl derivatives interfere. This indicates the desirability of quantitative separation of uric acid by precipitation. For this mercuric urate[14] is most satisfactory. Perchloric acid is the most satisfactory deproteinizing agent if one is to be used.[15]

Uric acid precipitated as a mercuric salt and redissolved in sodium chloride exhibits a maximum at 285 mμ. The mixture is read at 290 mμ to lessen background absorption. This permits determination of uric acid in the presence of xanthines and methyl-substituted uric acids present in plasma in concentrations below 0.2 mg. per ml. and in urine below 1 mg. per ml.[16] The protein precipitation is a critical step. If the procedure is not followed accurately, part of the uric acid is sorbed onto the protein. Beer's law is followed for 0.001-0.015 mg. per ml. The precipitation of uric acid with mercuric acetate is specific and quantitative. Cysteine or glutathione does not interfere.

Uric acid is oxidized to allantoin by manganese dioxide in an alkaline medium, hydrolyzed at 100° in an acid medium to glyoxylic acid, and developed by phenylhydrazine and ferricyanide to a red color.[17] Allantoin gives molybdenum blue with acid molybdate reagent.[18] Uric acid is read directly at 284 mμ.[19]

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[12] E. Praetorius and H. Poulsen, *Scand. J. Clin. and Lab. Invest.* **5**, 273-9 (1953); T. V. Feichtmeir and H. T. Wrenn, *Am. J. Clin. Pathol.* **25**, 833-9 (1955); C. A. Dubbs, F. W. David, and W. S. Adams, *J. Biol. Chem.* **218**, 497-504 (1956).

[13] H. M. Kalckar, *J. Biol. Chem.* **167**, 429-43 (1947).

[14] S. J. M. Auld, *Proc. Chem. Soc.* **23**, 151 (1907).

[15] C. Neuberg and E. Strauss, *Exptl. Med. & Surg.* **3**, 39 (1945).

[16] F. Bergmann and S. Dikstein, *J. Biol. Chem.* **211**, 149-53 (1954).

[17] F. Giannini and U. Zingoni, *Boll. soc. ital. biol. sper.* **29**, 1396-8 (1953).

[18] M. S. El Ridi, A. A. Magd, and El-Masry, *Proc. Pharm. Soc. Egypt, Sci. Ed.* **36**, No. 6, 71-7 (1954).

[19] A. Braschi and O. Cerri, *Boll. chim. farm.* **96**, 148-51 (1957).

Procedure—*By phosphotungstic acid reagent.*[20] *Serum or plasma.* To prepare the glycerine-silicate reagent, dissolve 10 grams of sodium silicate in 100 ml. of hot water and mix with 20 ml. of glycerine. Filter if cloudy. To prepare the phosphotungstic acid solution, reflux 50 grams of sodium tungstate, 400 ml. of water, and 40 ml. of 85% orthophosphoric acid for 4 hours. Dilute to 500 ml.

Mix 1 ml. of the sample, 8 ml. of water, and 0.4 ml. of 2% sodium hydroxide solution. Add 0.6 ml. of phosphotungstic acid reagent, adding the first half rapidly with stirring and the second half dropwise with stirring to ensure complete precipitation of protein. Filter after 5 minutes or centrifuge. To 10 ml. of the filtrate or centrifugate, add 5 ml. of glycerine-silicate reagent and 1 ml. of 2% sodium polyanetholsulfonate solution with constant mixing. With mixing add 1 ml. of phosphotungstic acid reagent and read after 15 minutes at 700 mμ. As a blank treat 10 ml. of standard and 10 ml. of water the same as the sample.

Alternatively, heat 0.2 ml. of a tungstic acid filtrate of the sample with 3 ml. of 4% sodium cyanide solution containing 15% of urea and 0.5 ml. of phosphotungstic acid reagent for 30 minutes at 35°. Cool and read at 690-700 mμ.

By arseno-18-tungstic acid.[21] *Serum or plasma.* Dilute a 2-ml. sample with 14 ml. of water. Add 2 ml. of 10% molybdenum-free sodium tungstate solution (Vol. III, p. 438), and 2 ml. of 1:59 sulfuric acid. Filter. To 5 ml. of the filtrate add 2.6 ml. of a sodium silicate-glycerine solution prepared by mixing 6 parts of 10% sodium silicate solution with 1 part of glycerine. Mix and add 0.4 ml. of arseno-18-tungstic acid reagent. Mix, let stand for 15 minutes, and read at 620 mμ.

By arsenophosphotungstic acid.[22] *Fresh, brackish, or sea water.* To prepare the arsenophosphotungstic acid reagent, dissolve 100 grams of sodium tungstate dihydrate in 600 ml. of water. Add 50 grams of arsenic pentoxide, 25 ml. of 85% phosphoric acid, and 20 ml. of concentrated hydrochloric acid. Bring the solution to a boil and add 20 ml. of saturated bromine water. Boil for 2 hours. Cool and dilute to 600 ml. Dilute a sample containing 0.02-0.12 mg. of uric acid to 30 ml. Add 20 ml. of 4% disodium dihydrogen ethylenediamine tetraacetate dihydrate solution as a buffer and to complex alkaline earth ions. Add 2.5 ml. of arsenophosphotungstic acid reagent and 4 ml. of 10% sodium cyanide solution. Mix and let stand until the maximum color forms, 10-60 minutes. Dilute to 50 ml., mix, and read at 890 mμ against water.

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[20] R. M. Archibald, *Clin. Med.* **3**, 102-5 (1957).

[21] E. R. Hausman, G. T. Lewis, and J. S. McAnally, *Clin. Chem.* **3**, 657-8 (1957).

[22] D. R. Norton, M. A. Plunkett, and F. A. Richards, *Anal. Chem.* **26**, 454-7 (1954).

By uricase and direct reading. Serum.[23] To test unknown uricase for activity, dilute so that 0.25 ml. contains sufficient enzyme to completely break down 0.1 mg. of uric acid under the test conditions. Use twice this amount. To prepare a glycine buffer, dissolve 50 grams of glycine in 400 ml. of water and add 200 ml. of 4% sodium hydroxide solution. Adjust the pH to 9.35 and dilute to 1 liter. Preserve the solution with a few drops of chloroform. As a reagent, add 0.5 mg. of Worthington uricase to 1 ml. of glycine buffer diluted 1:3 or dilute 1 ml. of Ido uricase 1:200.

To each of 2 tubes, add 2.5 ml. of sample, 0.5 ml. of buffer, and 4 ml. of water. To 1 tube add 1 ml. of uricase and incubate both for 2 hours at 45°. To stop the reaction add 4 ml. of 10% trichloroacetic acid solution to the tube containing the uricase and mix by inversion.

Prepare a mixture of 4 ml. of 10% trichloroacetic acid solution and 1 ml. of uricase and let stand for 20 minutes. Add this mixture to the blank and mix by inversion. Let both tubes stand for 20 minutes. Centrifuge for 15 minutes, longer if a clear upper layer is not obtained. With the spectrophotometer set at 294 mμ and the unknown set at zero, read the blank. This reading gives the decrease in optical density caused by action of uricase. To calculate the unknown:

$$\frac{\text{decrease in optical density of unknown}}{\text{decrease in optical density of standard}} \times \text{concentration of standard} = \text{unknown.}$$

Alternatively, to check the uricase activity, incubate 0.1 ml. of uric acid standard containing 0.04 mg. of uric acid in glycine buffer at pH 9.4 with 9.9 ml. of 0.04% uricase solution and read at 292 mμ in 10 minute intervals for three times. If the rate of decrease is not within the range of 0.0003 to 0.003 absorbance unit per minute, adjust it by dilution with buffer or addition of more uricase. To prepare this glycine buffer, dissolve 7.5 grams of glycine in 800 ml. of water and adjust the pH to 9.4 with 20% sodium hydroxide solution. Dilute to 1 liter.

Urine.[24] To 9.9 ml. of 0.04% uricase solution in glycine buffer, add a 0.1-ml. sample. Stopper, shake thoroughly, and read the initial absorbance immediately. Record the volume of the remaining sample-enzyme solution and unstopper. While heating at 45°, oxygenate for 10-20 seconds by passing a slow flow of oxygen through a constricted glass tube into the solution so that it is agitated without spattering. Continue to incubate for 4 to 16 hours at room temperature. Replenish any evaporated water, mix thoroughly, and read again.

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[23] T. V. Feichtmeir and H. T. Wrenn, *Am. J. Clin. Pathol.* 25, 833-9 (1955).

[24] C. A. Dubbs, F. W. Davis, and W. S. Adams, *J. Biochem.* 218, 497-504 (1956).

Subtract the final absorbance from the initial absorbance and correct by adding any absorbance of a uricase reagent blank. Divide by the factor k to give the uric acid concentration in mg. per ml. in the diluted sample. The factor k is the slope of the standard curve of absorbance change divided by concentration change and has a value near 0.075. If standard solutions and blanks are run together with the sample, place the sample, blank, and standard solution in cups hooked over the side of the reaction tube and after each series is set up, lower the cup into the tube so that premature uricase action is eliminated.

Separation as mercuric urate. Protein-free urine.[25] To prepare a wash liquid for the mercuric urate, add a solution of 10 mg. of uric acid in water to 1 liter of 0.3% mercuric acetate solution in 1% acetic acid solution. Heat for 1 hour at 80-90° to complete precipitation and let stand overnight. Filter and store.

Add 4 ml. dropwise during a 4-minute interval with continuous swirling to 1 ml. of 3% mercuric acetate solution in 5% acetic acid solution which is placed in a boiling water bath. Heat for 10 minutes, cool in ice water, and centrifuge for 10 minutes. Cautiously decant the supernatant liquid, removing the last part with a pipet. Disperse the precipitate in 5 ml. of the liquid wash. Centrifuge and remove the supernatant liquid as before. Add 8 ml. of 4% sodium chloride solution in 1% acetic acid solution and dissolve the mercuric urate precipitate by shaking. Filter if necessary and read at 290 mμ. Multiply the optical density by 1.55 to give the concentration of uric acid in mg. per ml. of undiluted urine. If the density is too high, dilute further with the sodium chloride reagent. If the initial concentration of uric acid is low, reduce the dilution of the sample.

Protein-containing urine. Dilute a sample 5 times and add a 3-ml. aliquot to 3 ml. of 6% perchloric acid solution. Filter and dilute the filtrate 5 times with 3% sodium tetraborate decahydrate solution. Proceed as for protein-free urine from "—add 4 ml. dropwise during. . . ."

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[25] F. Bergmann and S. Dikstein, *J. Biol. Chem.* **211,** 149-53 (1954).

Plasma in the presence of xanthines and their metabolites.[25] Centrifuge a 5-ml. heparinized or citrated, but not oxalated, blood sample for 10 minutes and dilute the plasma 5 times with water. To 10 ml. of 6% perchloric acid, add 10 ml. of sample dropwise with continuous swirling. Heat to 60° for 5 minutes, cool in ice, and filter. To 10 ml. of the filtrate add 2.5 ml. of 10% sodium tetraborate decahydrate in 7% sodium hydroxide solution. Mix and add 2 ml. of 3% mercuric acetate solution in 5% acetic acid solution to 10 ml. of the sample solution. Boil for 1 minute, and heat for 5 minutes at 100°. Centrifuge for 10 minutes and cautiously remove the supernatant liquid. Dissolve the precipitated mercuric urate in 8 ml. of 2% sodium hydroxide solution in 1% acetic acid solution and filter if necessary. Read at 290 mμ and multiply the result by 0.155 to calculate the mg. of uric acid per ml. of undiluted plasma. If values lower than 0.02 mg. per ml. of undiluted plasma are to be measured, use a larger sample and increase the volume of the reagents with the exception of the sodium chloride solution.

Plasma in the absence of xanthines and their metabolites. Follow the procedure for plasma in the presence of xanthines, reading the deproteinized sample directly at 290 mμ, eliminating the precipitation of uric acid from the plasma. Subtract a blank value of 0.04.

As allantoin. Tissue.[26] Homogenize a 15-gram sample with 75 ml. of 0.01% lithium carbonate solution and heat at 100° for 5 minutes. Add 15 ml. of 3% uranium acetate solution to 30 ml. of the homogenate and centrifuge. Add the supernatant liquid, approximately 37 ml., to 1 ml. of 40% sodium hydroxide solution. To the residue in the centrifuge tube, add 30 ml. of 0.5% uranium acetate solution and recentrifuge. Combine the supernatant liquids. Repeat starting from "To the residue in the centrifuge tube. . . ." The total supernatant liquids should not exceed 98 ml. If necessary adjust the pH to 13 with 40% sodium hydroxide solution. Dilute to 100 ml. and allow the yellow precipitate to settle. To 5 ml. of the supernatant liquid, add 0.3 ml. of a 0.1% suspension of manganese dioxide in 0.4% sodium hydroxide solution. Maintain at 37-40° for 40 minutes, agitating constantly to convert uric acid to allantoin. Filter, add 0.53 ml. of 5.3% sodium carbonate solution, and heat at 100° for 20 minutes. Let cool and adjust the pH to 2-3 with 1:10 hydrochloric acid. Heat for 2 minutes at 100°. Add 8 drops of 0.5% phenylhydrazine hydrochloride solution, shake, and cool. Add 4 drops of 5% potassium ferricyanide solution and 3 ml. of concentrated hydrochloric acid. Allow the red color to stand for 10 minutes, dilute, and read.

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[26] F. Giannini and U. Zingoni, *Boll. soc. ital. biol. sper.* 29, 1396-8 (1953).

GLUCURONIC ACID

Glucuronic acid is read at 430 mμ after reaction with sulfuric acid and urea. The method is not much affected by other sugars.[27] The accuracy of the determination of glucuronic acid with bis(4-aminophenyl) sulfone is within 10% and although the method is not as satisfactory as other methods, it has the advantage of not being affected by the presence of other neutral sugars.[28] An appropriate standard is butyrolactone.

The color developed in the naphthoresorcinol method (Vol. III, p. 442) can be developed in 30 minutes at 100°.[29] An alternative is to evaporate the sample and reagent to dryness, take up in 50% ethanol, and extract with toluene.[30] Glucose and ascorbic acid also react at lower sensitivity.

For determination by the color in 79% sulfuric acid see page 168. For determination by carbazole see page 197.

Procedure—*By sulfuric acid.* Dissolve 10 grams of urea in the minimum volume of water and make up to 200 ml. with 2:3 sulfuric acid. Add a 1-ml. sample containing about 0.5% of glucuronic acid to 20 ml. of the reagent and heat for 10 minutes at 100°. Cool for 1 minute in ice and read after 10-20 minutes at 430 mμ against 2:3 sulfuric acid.

By bis(4-aminophenyl) sulfone. To a 1-ml. sample add 5 ml. of 1% bis(4-aminophenyl) sulfone solution in 1:10 hydrochloric acid. Add 3 ml. of concentrated hydrochloric acid. Dilute to 10 ml. with water and heat at 100° for 30 minutes. Let stand for 35 minutes, filter, and read at 500 mμ.

By naphthoresorcinol. To a 2-ml. protein-free sample containing 0.02-0.15 mg. of glucuronic acid, add 4 ml. of a 1:1 mixture of 0.2% naphthoresorcinol solution and concentrated hydrochloric acid. Shake and heat at 98° for 30 minutes. Cool rapidly and extract with 4 ml. of butyl acetate. Reat at 570 mμ.

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[27] H. Kawabata, S. Tomioka, and K. Umeno, *Yakagaku Zasshi* **77**, 767-70 (1957).

[28] M. Ishidate, I. Kuriyama, and S. Takitani, *J. Pharm. Soc. Japan* **74**, 690-2 (1954).

[29] K. Heyns and C. Kelch, *Z. anal. Chem.* **139**, 339-51 (1953); T. Miettinen, V. Ryhanen, and H. Salomaa, *Ann. Med. Exptls. et Biol. Fenniae* **35**, 173-9 (1957).

[30] Masayuki Akashi, *Fukuoka-Igaku-Zassi* **48**, 2112-22 (1957).

Uronic Acids

Uronic acids produce a pink to red color with anthrone with a maximum absorbance at 540-550 mμ, increasing in color intensity until a maximum is reached after 44 hours.[31] Most carbohydrates react with the anthrone under the conditions of the procedure to give a blue-green color with a maximum absorbance at 620-625 mμ. *l*-Ascorbic acid produces a red color and 2-desoxy sugars yield colors with maxima in the 520-560 mμ range.

Uronic acids are also determined in carbohydrates with anthrone. Rhamnose, galactose, and galacturonic acid individually follow Beer's law at both 540 and 625 mμ. Mixtures of these carbohydrates also follow Beer's law at both wave lengths. The absorbances are additive. Sodium heparinate yields the characteristic uronic acid color with anthrone.

By dissolving the reagent in the same concentration of sulfuric acid there is no heat evolved when the sample and reagent are combined, eliminating the step of chilling. Tryptophan, methionine, and eysteine influence color development between uronic acids and anthrone as will be given in more detail. This applies to *l*-idurone, *l*-gulurone, *d*-mannurone, *d*-glucuronic acid, and *d*-galacturonic acid. Tryptophan produces its own color with anthrone but its spectrum can be distinguished from that of the glucuronic acid-anthrone spectrum. At 550 mμ the spectrum of tryptophan-anthrone and glucuronic acid-anthrone as well as that with mannurone-anthrone are additive, but the spectrum of idurone-anthrone is nonadditive. The influence of tryptophan can be reduced by reducing the temperature at which the color is developed from 100° to 70° since the absorbance of tryptophan in concentrations of 0.1-0.2 mg. per 6 ml. is 5 to 6 times greater at 100° than at 70°.

Methionine and cysteine do not produce colors with anthrone alone but influence the uronic acid color. Methionine may enhance the color at 70° by as much as 30% depending on the uronic acid being analyzed. When both cysteine and glucuronic acids are present in concentrations of 0.1-0.2 mg. per 6 ml., cysteine depresses the color intensity of the uronic acid-anthrone complex at 70° by 10% but increases the color intensity at 100°. Phenylalanine, tyrosine, proline, hydroxyproline, histidine, glutamic acid, lysine, valine, and arginine do not influence the anthrone color or produce colors of their own with anthrone.

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[31] J. R. Helbert and K. D. Brown, *Anal. Chem.* 28, 1098-1100 (1956); 29, 1464-6 (1957); 31, 1700-2 (1959).

Therefore, the estimation of uronic acids in the presence of large amounts of amino acids may be difficult, but when present in almost equal amounts the effects of tryptophan, methionine, and cysteine can be decreased by heating the reaction mixture to 70° instead of 100° and by decreasing the concentration of the sample.

Procedure—Dissolve the sample in 3:1 sulfuric acid. This will require about 30 minutes at room temperature for monomers and 60 minutes for polymers. To prepare the reagent, dissolve 0.16 gram of anthrone in 100 ml. of 3:1 sulfuric acid, allowing 1 hour for complete solution. To 4 ml. of the anthrone solution add 2 ml. of the sample in 3:1 sufuric acid. Heat at 100° for noncarbohydrate samples or at 70° for carbohydrate samples for 5-10 minutes. Cool at 4° for 3 minutes. Let stand 20 hours at room temperature and read at 550 mμ.

Nucleic Acids

p-Nitrophenylhydrazine measures the desoxyribose in desoxyribosenucleic acid.[32] The color develops on addition of the sodium hydroxide after which it starts to fade at the rate of 2% per minute over a 5-minute interval and at a slow rate thereafter. The *p*-nitrophenylhydrazine method shows a greater sensitivity than the diphenylamine method, but the former method on the average gives slightly lower values, and both methods exhibit a deviation of the order of 4-4.6%. These lower values may be explained by assuming that the *p*-nitrophenylhydrazine gives no color with protein or the hydrolysis products of protein. Beer's law is followed for 0.01-0.3 mg. of desoxyribosenucleic acid.

The method for the determination of desoxyribose by diphenylamine is modified by allowing the reaction mixture to stand overnight at room temperature rather than heating at 100° for 10 minutes.[33] This modification produces a more intense color, the procedure is 3.5 times as sensitive as the original method, and is not as influenced by interferences. Addition of acetaldehyde to the reagent is required when prepared with redistilled acetic acid. Perchloric acid counteracts the effect of any trichloroacetic acid present, and increases the rate of color development.

The following compounds do not develop color with the reagent:

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[32] J. M. Webb and H. B. Levy, *J. Biol. Chem.* 213, 107-17 (1955).

[33] K. Burton, *Biochem. J.* 62, 315-23 (1956).

sucrose, glucosamine, rhamnose, soluble starch, alginic acid, lactose, glucose, inositol, ascorbic acid, bovine plasma albumin, glutathione, cysteine hydrochloride, tryptophan, glycine, histidine hydrochloride, potassium gluconate, adenine sulfate, uric acid, adenosine-5′ phosphate, creatine hydrate, and chloral hydrate. The following substances in 2-mg. amounts give a detectable color which is less than that given by 0.0014 mg. of desoxyribonucleic acid: pyruvic acid, tannic acid, fructose, indole, ribose, arabinose, xylose, α-oxoglutaric acid, acid-hydrolyzed casein, and uracil. Dried human blood, glycogen, and commercial preparations of bile salts give readings equivalent to 6 millimicrogram atoms of desoxyribonucleic acid, 5 mg. of ribonucleic acid gives reading equivalent to 0.011 mg. of desoxyribonucleic acid, and 2 mg. of agar give a reading equivalent to 0.018 mg. of desoxyribonucleic acid.

The presence of 2 mg. of the following substances reduces the color: potassium gluconate, acid-hydrolyzed casein, tryptophan, ethylenediamine, and diethylenetriamine. Sodium chloride in concentration of 20 mg. per ml. reduces the color by 5% but the substances which have the greatest effect on reducing color are ascorbic acid, cysteine, and triethylenetetraamine. Glyceraldehyde and β-hydroxybutyraldehyde give some color with the reagent in the absence of desoxyribonucleic acid.

The sugars in both ribosenucleic acid and desoxyribosenucleic acid are determined by anthrone,[34] page 175. A maximum blue color for ribonucleic acid occurs at 620 to 625 mμ and a red color for desoxyribonucleic acid at 565 and 530 mμ. The color of the ribosenucleic acid disappears after heating for 10 minutes while the color from desoxyribosenucleic acid is unaffected. Since the absorbance values at 565 mμ and 620 mμ are additive, the nucleic acids can be determined in the presence of each other. Beer's law is followed for 0.1-0.4 mg. of desoxyribonucleic acid and for 0.1-0.5 mg. of ribonucleic acid. Pentoses and hexoses interfere.

p-Bromophenylhydrazine reacts with the acidified xylene-ethanol solution of furfural formed from the liberation of ribose from the ribonucleic acid. The color is stable and reproducible and is not formed by desoxyribosenucleic acid.[35] Although the hydrolysis of the nucleic acid can be accomplished in 15 minutes, a 30-minute hydrolysis is used to allow determination of the desoxyribonucleic acid in the same hydrolysate. Addition of sodium chloride enhances the furfural formation.

Essentially, only the purine-bound ribose is measured by this method as the pyrimidine nucleatides are not as easily hydrolyzed. Carbohydrates which yield furfural under the conditions of the procedure interfere. Galacturonic acid interferes. Glycogen, methyl glucoside, levulose, dextrose, and saccharose give some color with the reagent. Beer's law is followed over a range of 0.0094 to 0.15 mg. of ribonucleic acid.

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[34] N. D. Gary and R. E. Klausmeier, *Anal. Chem.* 26, 1958-60 (1954).

[35] Junius M. Webb, *J. Biol. Chem.* 221, 635-49 (1956).

Desoxyribose is determined by indole in a warm perchloric acid extract.[36] Of many structures ranging from 2-aminoacridine to rhodamines they are more strongly bound to desoxyribonucleic acid than to ribonucleic acid.[37]

The orcinol-ferric ion reaction absorbs at the same wave length with furfural, pentose nucleic acid, and desoxyribonucleic acid unless the acidity is reduced below 1:20 hydrochloric acid. Then the maximum absorption is at 590 mμ.[38] This orcinol and ferric ion reaction is ineffective in syrups or in the presence of lactose and potassium iodide. The reaction with cysteine is unsatisfactory in the presence of syrup, potassium iodide, and calcium ion.[39]

The nucleic acids are read in the ultraviolet after hydrolysis with trichloroacetic acid, which also acts as the protein precipitant.[40] Total nucleic acids are measured by this method. The total nucleic acids are also read in perchloric acid at 268 mμ.[41] Ribonucleic acid at pH 5.7 is best read at 260 mμ. Desoxynucleic acid is best read at pH 7.[42]

The usual methods for the determination of nucleic acids do not apply to egg yolks because of their very high phosphoprotein content. Fats are removed by treatment with acetone and ethanol in the cold and then by heating in diethyl ether and ethanol. The nucleic acids are isolated by extraction with 10% sodium chloride solution and precipitated with ethanol. Since the precipitate contains other phosphorus compounds in addition to nucleic acids, the solution is extracted with 5% trichloroacetic acid solution at 90° and the extract is extracted with ether. The nucleic acids are read at 260 mμ.[43] Nucleic acids are separable by paper chromatography.[44]

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[36] Giovanni Ceriotti, *Ibid.* **198,** 297-303 (1952); John Paul, *Analyst* **83,** 37-42 (1958).

[37] F. W. Morthland, P. P. H. DeBruyn, and N. H. Smith, *Exptl. Cell Research* **7,** 201-14 (1954).

[38] T. G. Brady and A. McEvoy-Bowe, *Congr. intern. biochim., Resumes communs., 2e Congr., Paris* **1952,** 198.

[39] Oscar Goglia, *Boll. chim. farm.* **91,** 309-12 (1952); Cf. Sam Brody, *Acta Chem. Scand.* **7,** 502-6 (1953).

[40] Junius M. Webb, *J. Biol. Chem.* **230,** 1023-30 (1958); Cf. O. P. Chepinoga, E. B. Skvirskaya, and L. P. Rukina, *Ukraïn. Biokhim. Zhur.* **23,** 335-40 (1951); Cf. Yvonne Moulé, *Arch. sci. physiol.* **7,** 241-54 (1953).

[41] John Paul, *Analyst* **83,** 37-42 (1958).

[42] S. W. Englander and H. T. Epstein, *Arch. Biochem. and Biophys.* **68,** 144-9 (1957).

Samples—*Tissues and microorganisms.*[45] In general, dehydrate all samples in ether and dry *in vacuo*. Add to 3 ml. of 5% trichloroacetic acid, 1-20 mg. of dry sample approximately equivalent to 6-120 mg. of fresh sample and containing 0.005-0.15 mg. of desoxyribosenucleic acid. Heat this suspension for 30 minutes at 100° to hydrolyze. Cool rapidly and add an equal volume of 5% trichloroacetic acid. Mix and centrifuge. Develop desoxyribonucleic acid by *p*-nitrophenylhydrazine or ribonucleic acid by *p*-bromophenylhydrazine.

Tissue.[46] Prepare a stock of salt solution by dissolving 80 grams of sodium chloride, 4 grams of potassium chloride, 1.4 grams of calcium chloride, and 2 grams of magnesium sulfate heptahydrate in 1 liter of water. For use dilute 1:9 with water.

Place the tissue in a centrifuge tube and after spinning discard the medium. Add 2 ml. of the salt solution, stir with a platinum wire, and centrifuge. Add 2 ml. of 3:1 ethanol-ether mixture and shake. Set aside for 15-20 minutes and centrifuge. Set aside the supernatant liquid. Again extract, using 2 ml. of 1:3 carbon tetrachloride-ether mixture. Repeat the standing and centrifugation. Take a third extract, this time using 2 ml. of 3:1 ethanol-ether. Combine the extracts as the lipid fraction for determination by a technic given on page 303.

Add 2 ml. of ice-cold 1:35 sulfuric acid to the residue in the tube. Mix and store at 0° for 10 minutes, stirring occasionally. Refrigerate while centrifuging and decant. Repeat this extraction twice. Combine the extracts as a sample for determination of carbohydrate and phosphorus.

Add 2 ml. of a 1:9 dilution of 60% perchloric acid to the residue in the tube. Stir and incubate at 70° for 20 minutes with occasional stirring. Centrifuge and decant. Repeat the extraction. The combined perchloric acid extracts contain the nucleic acid with some carbohydrate. Determine the nucleic acid by direct reading at 268 mμ, the desoxynucleic acid by indole.

The residue is largely protein. For its determination see Vol. IV, p. 170 *et seq.*

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[43] Zh. G. Schmerling and Kh. M. Ravikovich, *Doklady Akad. Nauk. S.S.S.R.* 93, 883-6 (1953).

[44] W. Kanngiesser, *Hoppe-Seyler's Z. physiol. Chem.* 291, 247-9 (1953).

[45] J. M. Webb and H. B. Levy, *J. Biol. Chem.* 213, 107-17 (1955).

[46] John Paul, *Analyst* 83, 37-42 (1958).

Bacteria.[47] Acidify a sample containing 0.00001-0.00015 mg. atom of desoxyribonucleic acid per ml. of culture with 25% perchloric acid solution so that the final concentration is 2.5%. Chill for 30 minutes and centrifuge. Break up the precipitate and stir with 0.5 ml. of 5% perchloric acid solution. Add 3.5 ml. of 5% perchloric acid and heat at 70° for 15 minutes with occasional stirring. Centrifuge, decant the supernatant liquid, and re-extract the precipitate with an additional 3 ml. of 5% perchloric acid. Develop a portion of the combined extracts with diphenylamine.

Procedure—*By* p-*nitrophenylhydrazine.*[48] Add 2 ml. of 5% trichloroacetic acid to a 2-ml. aliquot of sample containing 0.01-0.3 mg. of desoxyribosenucleic acid. Add 0.2 ml. of 0.5% *p*-nitrophenylhydrazine solution in 95% ethanol. Heat for 20 minutes at 100°, cool rapidly, and add 10 ml. of *n*-butyl acetate to extract excess reagent. Stopper and shake for 5 minutes. Centrifuge and discard the organic layer. Add 1 ml. of 8% sodium hydroxide solution to 3 ml. of the aqueous phase and dilute to 5 ml. with water. Read against water at 560 mμ.

By diphenylamine.[49] As a reagent dissolve 1.5 grams of diphenylamine in 100 ml. of redistilled acetic acid, add 1.5 ml. of concentrated sulfuric acid and store in the dark. Before use add 0.1 ml. of 1.6% acetaldehyde solution for each 20 ml. of reagent.

Dilute the nucleic acid extract in perchloric acid solution with 5% perchloric acid solution so that the final solution contains 0.00002-0.00025 mg. atom of desoxyribonucleic acid per ml. Mix 2 ml. with 4 ml. of diphenylamine reagent and incubate at 30° for 16-20 hours. Read at 600 mμ against 5% perchloric acid solution.

By anthrone.[50] Dilute a sample to 2 ml. with water and cool in ice. With the tube in an inclined position add 4 ml. of cold 0.2% anthrone solution in concentrated sulfuric acid so that the anthrone forms a layer below the sample. Mix thoroughly by shaking while still in the ice bath. Cover and heat at 100° for 2.5 minutes for ribonucleic acid and 10 minutes for desoxyribonucleic acid. Cool to room temperature rapidly in ice and read against a reagent blank containing 2 ml. of water and 4 ml. of anthrone reagent at 565 and 620 mμ. The reading at 565 mμ is that of desoxyribonucleic acid, while the reading at 620 mμ is a total of both nucleic acids. Calculate the desoxyribonucleic acid at 620 mμ by multiplying the value measured at 565 mμ by a conversion factor K. K is equal to the ratio of the absorbance value with standard solutions of desoxyribonucleic acid at 620 mμ and 565 mμ after reaction periods of 2.5 and 10 minutes respectively or $K = DNA_{620}/DNA_{565}$.

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[47] K. Burton, *Biochem. J.* 62, 315-23 (1956).

[48] J. M. Webb and H. B. Levy, *J. Biol. Chem.* 213, 107-17 (1955).

[49] K. Burton, *Biochem. J.* 62, 315-23 (1956).

[50] N. D. Gary and R. E. Klausmeier, *Anal. Chem.* 26, 1958-60 (1954).

By p-*bromophenylhydrazine.*[51] To prepare the *p*-bromophenylhydrazine hydrochloride solution, dissolve 2.5 grams of the hydrochloride in a solvent containing 2 ml. of concentrated hydrochloric acid and 100 ml. of 95% ethanol. Add 1 gram of activated carbon per 30 ml. of reagent to decolorize the solution. Prepare daily.

To 1 ml. of a diluted hydrolyzed sample containing 0.009-0.2 mg. of ribonucleic acid in 5% trichloroacetic acid solution, add 1 ml. of 2:1 hydrochloric acid, 1 ml. of xylene, and enough sodium chloride crystals to saturate the mixture. Cover and heat at 100° for 3 hours. Cool, add 2 ml. of xylene, mix, and centrifuge.

To 2 ml. of the xylene layer containing the furfural formed from the ribose add 2 ml. of *p*-bromophenylhydrazine reagent. Mix, stopper, and incubate at 37° for 1 hour. Dilute to 5 ml. with 1:50 mixture of 1:2 hydrochloric acid and 95% ethanol. Read at 450 mμ against a blank containing 1 mg. of ribonucleic acid in 1 ml. of 5% trichloroacetic acid solution.

By indole.[52] *Desoxynucleic acid.* To a 2-ml. aliquot of the perchloric acid extraction of the sample containing 0.005-0.03 mg. of desoxyribonucleic acid, add 1 ml. of 0.04% indole solution and 1 ml. of concentrated hydrochloric acid. Heat at 100° for 10 minutes. Cool and extract three times with 4-ml. portions of chloroform. Discard the chloroform extracts and centrifuge the remaining solution at a low speed to remove any emulsion. Read the yellow color at 490 mμ.

Direct reading.[53] *Tissue.* Heat the tissue powders at 100° for 30 minutes with 5% trichloroacetic acid solution. Dilute with an equal volume of unheated 5% trichloroacetic acid solution. Dilute a 1-ml. aliquot containing the equivalent of 0.1 mg. of total nucleic acids to 5 ml. with water. Read against 5% trichloroacetic acid solution at 265 mμ, except read yeast at 263 mμ since it is predominantly ribonucleic acid.

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[51] Junius M. Webb, *J. Biol. Chem.* 221, 635-49 (1956).

[52] John Paul, *Analyst* 83, 37-42 (1958).

[53] Junius M. Webb, *J. Biol. Chem.* 230, 1023-30 (1958).

Alternatively, separate the ultraviolet-absorbing components from the trichloroacetic acid medium as follows. Heat the sample at 100° for 30 minutes with 5% trichloroacetic acid solution. Neutralize a 1-ml. aliquot containing the equivalent of 0.1 mg. of total nucleic acid with 2 drops of concentrated ammonium hydroxide. Heat for 15 minutes at 100° to expel excess ammonia and cool to 0-5°. Add 1 ml. of cold 2% silver nitrate solution, mix, and let stand for 15 minutes in an ice bath. Centrifuge and wash the residue with cold 1% silver nitrate solution. Recentrifuge. Add 4 ml. of 1:110 hydrochloric acid to the residue, cover, and heat for 10 minutes at 100°. Cool, centrifuge, and dilute a 2-ml. portion of the supernatant liquid to 5 ml. with water. Read against trichloroacetic acid solution at 269-272 mμ.

Desoxyribonucleic Acid

The reaction of indole with desoxyribonucleic acid gives a pink color read in chloroform at 490 mμ.[54] Beer's law is followed at 0.0025-0.015 mg. per ml. There is no interference by glucosamine, sorbitol, levulinic acid, uric acid, creatine, and creatinine at 0.1 mg. per ml. Casein hydrate at 1 mg. per ml. gives an extractable yellow color. Colors absorbing at 490 mμ and extractable by chloroform are given by ribose, levulose, glucose, xylose, sucrose, mannose, lactose, galactose, sorbose, and ascorbic acid. The color with arabinose is only partially extractable.

As an alternative heat a solution of the order of 0.0125 mg. per ml. with 5% trichloroacetic acid for 15 minutes at 90° to hydrolyze to desoxyribose. Determine the latter by periodic acid and 2-thiobarbituric acid, page 177.

Procedure—To 2 ml. of sample solution add 1 ml. of 0.04% indole solution and 1 ml. of concentrated hydrochloric acid. Heat at 100° for 10 minutes and cool. Extract with 4,4, and 4 ml. of redistilled chloroform. Read the combined chloroform extracts at 490 mμ.

Pyrethrins

The method of determination of pyrethrin with acid mercuric sulfate (Vol. III, pp. 464-6) is applicable only to the determination of pyrethrin I and estimates allethrin as well.[55] Dilution of the reagent avoids precipitation and acts as a stabilizer to permit longer storage of the reagent. A partial irreversible adsorption and consequently an incomplete extraction is encountered with adsorptions on paper and on fritted glass, especially when petroleum ether is used as the solvent. In these cases when the extracted substrate is treated with the reagent, the color formed indicates incomplete extraction even before the rest of the analysis is completed. In such cases higher boiling solvents such as dioxane and butanol are used and the extraction is forced by adding a soluble acid such as acetic acid and a surface-active agent such as dodecylbenzenesulfonic acid. Even with this modified procedure, a satisfactory extraction is not always obtained because of the influence of sizing and coating additives in the sample which exert a strong adsorptive power. Interfering substances such as cyclopentenolone and other fatty acid esters can be removed by saponification, barium chloride precipitation, and filtration. The method is applicable to determination of pyrethrins and allethrin in DDT, synergist MGK 264 (98% N-2-ethylhexylbicyclno[2.2.1]-5-heptene-2,3-dicarboximide), piperonyl butoxide (80% α-[2-(2-butoxyethoxy) ethoxy]-4,5-methylenedioxy-2-propyltoluene and 20% related compounds), Lethane 384 (50% β-butoxy-β-thiocyanodiethyl ether), oleic acid, or rancid corn oil. Beer's law is followed for 0.02-0.2 mg. of chyrsanthemic acid.

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[54] Giovanni Ceriotti, *Ibid.* **198,** 297-303 (1952).

[55] A. A. Schreiber and D. B. McClellan, *Anal. Chem.* **26,** 604-7 (1954).

Both pyrethrin I and II produce a red color with a mixture of orthophosphoric acid and ethyl acetate which follows Beer's law up to 0.1 mg.[56] Cyclethrin, pyrethrolone, furethrin, and ryanodine produce color with the reagent while toxaphene produces a turbidity. Cyclethrin can be distinguished from pyrethrin as it takes a longer period to develop the color. If piperonyl butoxide is present in excess such as a ratio of 10:1, the color development is suppressed. The color is also reduced if sulfoxide is present in excess of 5:1. Pyrethrins must be separated from these interferences by chromatography. Allethrin, natural chrysanthemum dicarboxylic acid, synthetic cinerin I, cinerolone, technical allethrolone, technical chrysanthemum monocarboxylic acid, rotenone, lindane, methoxychlor, DDT, chlordane, dieldrin, and aldrin do not form a color with the reagent.

A red color develops when pyrethrins are treated with sulfur solutions in alcoholic potassium hydroxide and in carbon tetrachloride.[57] Beer's law is followed up to 1.4 mg. of pyrethrins per ml. when a 560-mμ band is used and up to 0.2 mg. of pyrethrins per ml. when a 440-mμ band is used. The optimum range for the 560-mμ band is 0.3-1.2 mg. per ml. and for the 440-mμ band is 0.05-0.15 mg. per ml. Acetaldehyde, ethyl chrysanthemumate, and allethrolone develop color with the reagent. Compounds normally found in commercial insecticides, such as synergists and DDT, do not interfere. Kerosene also does not interfere.

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[56] H. L. Williams, W. E. Dale, and J. P. Sweeney, *J. Assoc. Offic. Agr. Chemists* **39**, 872-9 (1956).

[57] Louis Feinstein, *Science* **115**, 245-6 (1952); L. W. Levy and P. E. Estrada, *J. Agr. Food Chem.* **2**, 629-32 (1954).

Pyrethrin is read in the ultraviolet after extraction from pyrethrum flowers and powders with petroleum ether. The interference of kerosene is removed by vacuum distillation at 40° as higher temperatures cause decomposition of pyrethrin. Unsaturated compounds including aldehydes, ketones, and carboxylic acids interfere. Rubber stoppers and connections should not be used in the apparatus. The method is applicable for 0.1-1.5 mg. of total pyrethrin per 100 ml.[58] Sodium sulfide is also a reagent for pyrethrins, page 431.

Samples—*Flour from pyrethroid treated bags.*[59] Extract a 5-10 gram sample with three 20-ml. portions of petroleum ether. Filter the combined petroleum ether phases and develop all or an aliquot with mercuric sulfate. Thus after concentrating this extract somewhat it is appropriate for refluxing with 3-5 ml. of 0.2% sodium hydroxide solution in ethanol in that procedure.

Impregnated paper or cloth bags. A suitable sample should contain the equivalent of 0.05-0.1 mg. of chrysanthemic acid. Extract three to four times with 15-ml. portions of 1-butanol at 70° for 45 minutes each. Concentrate the combined extracts to approximately 2 ml. by slow vacuum distillation at 45° and 35 mm. of mercury. Use this as the aliquot for development by mercuric sulfate starting with dilution to 5 ml. with hexane.

Insecticide dusts. If the pyrethrins or allethrin have been deposited on the extender materials such as talcum, pyrophyllite, diatomaceous earth, etc., follow the procedure for allethrin or pyrethrin I in paper and cloth bags. If the allethrin or pyrethrin I have not been deposited on the extender materials, follow the procedure for flour taking an aliquot representing about 0.1 mg. of pyrethrins or allethrin in either case for development by mercuric sulfate.

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[58] A. J. Shukis, D. Cristi, and H. Wachs, *Soap Sanit. Chemicals* **27**, No. 11, 124-7 (1951).

[59] A. A. Schreiber and D. B. McClellan, *Anal. Chem.* **26**, 604-7 (1954).

Kraft paper, wool, cotton cloth, grains, burlap, and cardboard.[60] Grind the sample and extract with hexane for 1 hour using 2 ml. of solvent for each gram of sample. Concentrate an aliquot of the extract containing 0.025-0.1 mg. of pyrethrins to 25-35 ml. at 100°. Transfer to a test tube and evaporate at 100° to dryness, completing the final stages of evaporation out of the water bath by allowing the solution to come in contact with the sides of the tube as the pyrethrins should not be overheated. If the sample contains appreciable amounts of oil or color, add 0.2 ml. of odorless kerosene. Develop with orthophosphoric acid-ethyl acetate.

Pyrethrum flowers and powders. To an extraction thimble add a weighed sample containing 20-40 mg. of pyrethrin. Stopper with a similar shortened thimble having pin holes in the lower end. Extract with hexane for about 6 hours. Let stand overnight for precipitation of the wax. Filter and dilute to 100 ml. with hexane. Remove the solvent from a 2-ml. aliquot at 40° and apply a vacuum of 3 mm. for 15 minutes, using a ground glass joint adapter to connect the flask to the vacuum. Read the residue in the ultraviolet.

Alternatively, extract a ground sample with hexane for 6 hours. Remove the petroleum ether *in vacuo* from an aliquot and dissolve the solvent-free residue in kerosene to obtain a solution of 0.2-1 mg. of pyrethrins per ml. Develop a 1-ml. aliquot with sulfur-potassium hydroxide and sulfur-carbon tetrachloride.

Extracts and oil-based insecticides. Dissolve a weighed sample in kerosene so that it contains 0.2-1 mg. of pyrethrins per ml. Develop with sulfur-potassium hydroxide and sulfur-carbon tetrachloride.

Procedure—*By mercuric sulfate.*[61] As a reagent mix 1 gram of yellow mercuric oxide with 80 ml. of water. While stirring in a cold water bath, slowly add 40 ml. of concentrated sulfuric acid and stir until dissolved.

Dilute a sample solution to 100 ml. with hexane. Reflux an aliquot containing 0.1 mg. of chrysanthemic acid for 30 minutes to 1 hour with 3-5 ml. of 0.4% sodium hydroxide solution in 95% ethanol. Add 10 ml. of water, boil off the ethanol, and cool. Add 1 ml. of 10% barium chloride solution, mix, and let settle for several minutes. Filter, rinse twice with water, and strongly acidify the filtrate with 1:4 sulfuric acid. Filter through paper coated with Celite and extract the filtrate three times with 10-15 ml. of hexane. Evaporate the combined hexane layers to approximately 2 ml. at 100°. Cool immediately. Do not evaporate to dryness. Take up in small portions of hexane, not exceeding a total of 5 ml. Carefully evaporate the solvent, avoiding dryness as overheating may drive off some of the chrysanthemic acid. Cool rapidly, add 5 ml. of reagent, and stopper. Shake and centrifuge for 30-45 seconds to remove air bubbles. Read at 540 mμ at 1-minute intervals against 5 ml. of reagent and record the maximum which should be reached in 2-5 minutes. The method does not distinguish between pyrethrin I and allethrin.

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[60] H. L. Williams, W. E. Dale, and J. P. Sweeney, *J. Assoc. Offic. Agr. Chemists* 39, 872-9 (1956).

[61] A. A. Schreiber and D. B. McClellan, *Anal. Chem.* 26, 604-7 (1954).

By orthophosphoric acid-ethyl acetate.[62] To a residue containing 0.025-0.1 mg. of pyrethrins, add 5 ml. of a 4:1 mixture of 85% orthophosphoric acid and ethyl acetate. Shake for 1 minute and heat at 100° for exactly 3 minutes. Centrifuge for 15 minutes or until no bubbles remain in the solution. Cool to room temperature and read at 550 mμ against a 5-ml. reagent blank.

In the determination of kraft paper, wool and cotton cloth, compare directly with the standard. With materials such as grains, burlap, and cardboard which contain substances that suppress pyrethrum color, add a known amount of pyrethrin standard to extracts of untreated samples to use as standards.

By sulfur-potassium hydroxide and sulfur-carbon tetrachloride. To prepare the sulfur-potassium hydroxide reagent, mix 0.25 gram of flowers of sulfur with 1 liter of 5.6% potassium hydroxide solution in anhydrous ethanol. Let stand, usually overnight, with occasional shaking, until dissolved. If there is faint cloudiness, centrifuge before using and keep protected from air and moisture. To prepare the sulfur-carbon tetrachloride reagent, mix 2.5 grams of flowers of sulfur with 1 liter of carbon tetrachloride and let stand with occasional agitation until dissolved. If the flowers of sulfur do not dissolve completely in carbon tetrachloride this reagent is prepared by extracting flowers of sulfur with carbon tetrachloride, determining the total solids, and diluting to a final concentration of 2.5 grams per liter.

To a 1-ml. aliquot containing 0.2-1 mg. of pyrethrins, add 3 ml. of 0.25% sulfur solution in carbon tetrachloride. Add 3 ml. of sulfur-potassium hydroxide solution. Shake for 15 seconds and note the time of mixing. Stopper and develop at 30° for exactly 73 minutes from mixing time. Add a small amount of Celite filter aid and filter within 2 minutes. Shake the filtrate and read exactly 75 minutes after the time of mixing against 1 ml. of kerosene similarly treated. For pyrethrins between 0.2 and 1 mg. per ml. read at 560 mμ. For pyrethrins between 0.04 and 0.2 mg. read at 440 mμ. In the latter case the colorimetric readings are about three times larger than those obtained at 560 mμ.

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[62] H. L. Williams, W. E. Dale, and J. P. Sweeney, *J. Assoc. Offic. Agr. Chemists* 39, 872-9 (1956).

To correct for the color of the extract, add 3 ml. of carbon tetrachloride to 1 ml. of the pyrethrum extract, followed by 3 ml. of 5.6% potassium hydroxide solution in anhydrous ethanol. Shake for 15 seconds and develop at 30° for 73 minutes. Follow the procedure, starting at ". . . shake for 15 seconds and note the time of mixing."

Direct reading.[63] The sample should have been taken to dryness at not over 40°. Dissolve the residue containing 0.1-1.5 mg. of total pyrethrins per 100 ml. in 30 ml. of SD 3A ethanol. Shake 5 minutes and dilute to 100 ml. with the solvent. Read at 227 mμ against the solvent.

By 2,4-dinitrophenylhydrazine. See page 432.

By sodium sulfide. See page 433.

ALLETHRIN

Allethrin, *dl*-2-allyl-4-hydroxy-3-methyl-2-cyclopentene-1-one ester of *dl-cistrans*-chrysanthemum monocarboxylic acid, converted to the 2,4-dinitrophenylhydrazone derivative is analyzed chromatographically on silicic acid and the main band is determined photometrically at 375 mμ. The entire allethrin molecule is used in the formation of the hydrazone rather than just the acid part formed after cleavage. The results with this method are accurate to about ±10%. The sensitivity can be increased at least twofold by adding alcoholic alkali to the final 2,4-dinitrophenylhydrazone solutions and reading at 435 mμ.

It is possible to determine allethrin in mixtures containing up to 5% of each of the three acidic contaminants appearing in technical allethrin, chrysanthemum-monocarboxylic acid, its anhydride, and its acid chloride.[64] Piperonyl cyclonene, pyrethrins, and cyclethrin interfere. Beer's law is followed for absorbance values up to 0.6.

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[63] A. J. Shukis, D. Cristi, and H. Wachs, *Soap. Sanit. Chemicals* 27, 124-7 (1951).

[64] N. Green and M. S. Schechter, *Anal. Chem.* 27, 1261-5 (1955).

The hydrazone may also be passed through an alumina column. Beer's law is followed up to 60 mg. of allethrin and the optimum range is 25-45 mg.[65] Since both allethrin and pyrethrins give the same reaction with dinitrophenylhydrazine, this is also a method for pyrethrins. Allethrin equivalents from a calibration curve prepared with pure allethrin are convertible into pyrethrin equivalents by multiplying by a molecular weight factor of 1.07.

Allethrin, pyrethrins, and furethrin all react with sodium sulfide. Therefore it must be established which compound is present in the sample as the method does not distinguish between them.[66] The chrysanthemic acid ester of 2-benzyl-4-hydroxy-3-methyl-2-cyclopentene-1-one and the ester of 4-hydroxy-2-phenyl-3-methyl-2-cyclopentene-1-one form a deep red color with the reagent, isophorone a dark red, cinerins a dark red, allethrone a dark orange vermilion, chrysanthemic acid ester of 4-hydroxy-2-(*p*-methoxybenzyl)-3-methyl-2-cyclopentene-1-one a dark orange vermilion, and the *n*-propyl isomer a light yellow.

The following compounds do not form a color with the reagent: butyl oleate, butyl stearate, casein, chrysanthemic acid, chrysanthemic anhydride, chrysanthemic dicarboxylic acid, DDT, ethyl laurate, lindane, methoxychlor, oleic acid, piperonyl butoxide, piperonyl chrysanthemate, sesame oil, starch, stearic acid, and sulfoxide (*n*-octylsulfoxide of isosafrole). Beer's law is followed for 0.5-1 mg. Allethrin is also read in the infrared at 5.81μ.[67]

Samples—*Paper and cloth bags.* Extract a 0.5 square foot sample cut into strips for 3 hours with 125 ml. of acetone. Concentrate the extract at 100° to approximately 50 ml. and transfer quantitatively into 150 ml. of 5% sodium chloride solution. Add 15 ml. of hexane, shake for 1 minute, allow the layers to separate, and re-extract the aqueous layer twice with 5-ml. portions of hexane. Wash the combined hexane extracts with 15 ml. of water, draw off the lower aqueous layer, and allow the extract to drain through a cotton plug previously wet with hexane. Rinse the funnel with 10 ml. of hexane and drain through the cotton plug. Dilute to 50 ml. Evaporate the solvent to dryness at 70°. Add 10 ml. of 95% ethanol and swirl to dissolve. Develop with sodium sulfide.

Miscellaneous. For extraction of a variety of types of sample ranging from grains to insecticidal dusts see pyrethrins.

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[65] B. P. Moore, *J. Sci. Food Agr.* **5**, 500-504 (1954).

[66] C. Cueto and W. E. Dale, *Anal. Chem.* **25**, 1367-9 (1953).

[67] Stanley K. Freeman, *Ibid.* **27**, 1268-74 (1955).

Procedure—*By 2,4-dinitrophenylhydrazine.* The 2,4-dinitrophenylhydrazine reagent should be recrystallized from or extracted several times with hot benzene to remove impurities. The solvents should be purified from aldehydes or ketones by refluxing with 2,4-dinitrophenylhydrazine and distilled before use. Use a stopcock grease insoluble in benzene. Prepare a reagent solution by refluxing 250 mg. of dinitrophenylhydrazine with 28 ml. of anhydrous methanol containing exactly 0.10 ml. of concentrated hydrochloric acid. Cool to room temperature and add 14 ml. of reagent to a sample containing approximately 100 mg. of allethrin. Shake mechanically for 2.5 hours. Dilute with 60 ml. of water at 5° and extract with 20 ml. or more of benzene. Allow the layers to settle. Extract the aqueous layer with 15 ml. of benzene in a second funnel and with 10-ml. portions of benzene in a third and a fourth funnel. Combine the first three extracts in the first funnel, drain off any water, and filter through a cotton plug which has been washed with acetone and dried at 80° into the third funnel. This removes most of the excess dinitrophenylhydrazine. Rinse the two empty funnels with the fourth portion of benzene and filter through the cotton plug into the third funnel. Rinse each empty funnel with two 5-ml. portions of benzene and filter each rinsing into the third funnel. Wash the combined extract and rinsing twice with 15 ml. of saturated sodium chloride solution. Drain off the sodium chloride solution and filter the benzene. Rinse the funnel with benzene, filter into the extract, and dilute to 200 ml. with benzene.

Prepare a thin slurry of 20-22 grams of a 2:1 mixture of silicic acid and Hyflo Super-Cel. Mix thoroughly and pour rapidly into a 25 mm. × 400 mm. chromatograph column. Remove air bubbles in the column by stirring. Wash the walls of the column with a few ml. of fresh benzene and pack the slurry by gentle tapping and application of a moderate pressure from a nitrogen cylinder. The depth is about 120 mm. Drain most of the liquid through the column to within 5 mm. of the top of the adsorbant and introduce a 5-ml. aliquot of the sample into the column so that it drains down the walls. Rinse the beaker containing the sample and the dropper used for transfer with 1 ml. of a 5:5:3 mixture of benzene, hexane, and ether and add to the column. Allow the liquid to flow into the adsorbant with the aid of gentle pressure from a nitrogen cylinder. Rinse the beaker and dropper with 2-3 portions of mixed solvent, allowing each rinsing to enter the adsorbant before adding the next rinsing. Fill the column with the mixed solvent and apply a gas pressure of 25-30 cm. of water. Do not allow the column to run dry or channeling will occur.

Allow approximately 40 ml. of mixed solvent to drain from the column. At this point the first band containing the bulk of the material will approach the outlet. Three or more bands are usually visible. The first band is usually wide and diffuse and may appear heterogeneous possibly owing to the formation of syn- and antiforms of dinitrophenylhydrazones. Elute the first band from the column with 60-80 ml. of mixed solvent until the second band approaches the outlet. Dilute to 100 ml. and dilute further if necessary to bring the solution within a range where it can be read in a photometer. Read at 375 mμ.

Alternatively, dissolve a sample containing about 20 mg. of α-allethrin in 15 ml. of 1:9 sulfuric acid in carbonyl-free ethanol. Add 80 mg. of powdered 2,4-dinitrophenylhydrazine and swirl gently to dissolve. Stopper and heat at 80° for 20 minutes. Cool, take up in 20 ml. of ether, and shake with 15 ml. of water. Separate the aqueous layer and wash the ether with 15 ml. of water followed by 10 ml. of water. Re-extract the combined aqueous layer and washings with 20 ml. of ether and wash the ether layer with two 10-ml. portions of water. Repeat the extraction with another 20-ml. portion of ether. Concentrate the combined ether layers and dilute the residue with 25 ml. of dry benzene. Distill at 100° until the dry benzene begins to pass over. Cool and dilute with an equal volume of dry hexane.

Neutralize alumina by washing with dilute hydrochloric acid and water. Dry at 80° and standardize to grade III activity. Prepare a slurry of 50 grams of grade III alumina in 1:1 benzene-hexane and add to a 2.5-cm. chromatographic tube. Pack evenly and add the dinitrophenylhydrazone solution. Develop the chromatogram with the benzene-hexane solvent. Free 2,4-dinitrophenylhydrazine appears as a dark band at the top and the orange allethrin derivative moves slowly down the column. When the allethrin derivative reaches the bottom, elute with 2:1 benzene-hexane. Free the eluate from the bulk of the hexane *in vacuo* and dilute to 100 ml. with benzene. Dilute a 10-ml. aliquot to 100 ml. with benzene and read with a blue-green filter against benzene.

By sodium sulfide. To an ethanolic solution containing 0.5-1 mg. of pyrethrin, allethrin, or furethrin, add 1 ml. of 1% sodium sulfide pentahydrate solution and mix gently. Observe the time. Heat at 70° ±5° for exactly 15 minutes and add 0.3 gram of Hyflo Super-Cel. Agitate by vigorous tapping and immerse in water at 25° ± 5°. Filter into a cuvet exactly 20 minutes after addition of reagent and read the orange to red color at 540 mμ 25 minutes after addition of the reagent. Read against a reagent blank containing 1 ml. of reagent and 10 ml. of 95% ethanol similarly treated. Correct for an untreated sample carried through the procedure.

HEPARIN

Heparin forms a red precipitate with Toluidine Blue O (Vol. III, pp. 467-8). This is either read in alkaline ethanolic solution at 530 mμ,[68] or the dye solution is read at 245 mμ or 255 mμ before and after precipitation of the dye.[69] The solution of the dye in alkali can also be read at 280 mμ. Heparin in the range of 0.01-0.04 mg. is precipitated from blood plasma with *n*-octylamine hydrochloride and reprecipitated with zinc hydroxide The precipitate is dissolved in phosphate buffer and the final red precipitate is formed on addition of the dye (Cf. Vol. III, pp. 467-8). The heparin-protamine compound is read turbidimetrically.[70]

Procedure—*Blood plasma.* Dilute a 5-ml. oxalated sample with 5 ml. of $M/15$ phosphate buffer at pH 6.1 (Vol. I, p. 174). Prepare a solution of 2 ml. of *n*-octylamine hydrochloride, 1 ml. of concentrated hydrochloric acid, and 30 ml. of water. Adjust the pH to 7.3-7.5 and add 2 ml. of this solution in 0.5-ml. portions at 5-minute intervals to the sample. Centrifuge as soon as flocking is observed. If flocking does not occur in 10 minutes, heat to 40°. Centrifuge and wash the precipitate by suspension in 10 ml. of water. Centrifuge, moisten the precipitate with 0.5 ml. of 2% sodium hydroxide solution and 10 ml. of 0.9% saline solution, and heat at 60-70° for 15 minutes. Cool, add 0.5 ml. of 8% zinc sulfate heptahydrate solution, mix, and let stand for 30 minutes. Centrifuge and extract the heparin residue with 5 ml. of water and 5 ml. of phosphate buffer at pH 7.8 (Vol. I, p. 174). Heat at 65-70° for 15 minutes and centrifuge. The heparin is now in the supernatant layer.

Prepare a reagent by dispersing 100 mg. of Toluidine Blue O in 100 ml. of water. Dilute to 250 ml. and centrifuge. To a solution containing 0.01-0.04 mg. of heparin, add 1 ml. of reagent and let stand several hours or overnight. Centrifuge the red precipitate, drain, and wash with 5 ml. of water. Centrifuge and drain. Swab the inside of the tube with an ethanol-moistened and a dry gauze sponge to remove adhering dye. Add

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[68] R. B. Gibson, T. L. Carr, S. Green, W. M. Fowler, and I. Jenson, *Proc. Soc Exptl. Biol. Med.* 79, 577-80 (1952); Cf. W. Awe and K. D. Stüdemann, *Arzneimittel-Forsch.* 8, 98-9 (1958).

[69] W. Awe and K. D. Stüdemann, *Arzneimittel-Forsch.* 8, 98-9 (1958).

[70] G. Fekete, *Naturwissenschaften* 43, 279-80 (1956).

4 ml. of 95% ethanol and 1 ml. of 0.4% sodium hydroxide solution. Centrifuge after 20-30 minutes. Read at 530 mμ against a blank containing 5 ml. of water and 5 ml. of pH 7.8 phosphate buffer treated with Toluidine Blue O as above.

SODIUM CARBOXYMETHYLCELLULOSE, *CMC*

Sodium carboxymethylcellulose is precipitated as the copper salt to separate it from penicillin, procaine penicillin, dihydrostreptomycin, and streptomycin. It is then hydrolyzed to glycolic acid with sulfuric acid and read at 530 mμ after reaction with 2,7-naphthalenediol[71] (Cf. Vol. III, p. 467). The degree of substitution on each sample of sodium carboxymethylcellulose affects the color and this must be considered in the preparation of standard curves. Beer's law is followed.

Samples—*Pharmaceutical preparations.* Mix a sample containing 2-4 mg. of sodium carboxymethylcellulose with 50 ml. of water until dissolved. Adjust the pH to 2.5 with 1:9 hydrochloric acid and add 50 ml. of 1% cupric sulfate pentahydrate solution. Stir and adjust the pH to 4.1 by dropwise addition of 1:9 ammonium hydroxide solution. Let stand for 1 hour to allow the copper carboxymethylcellulose to settle. Decant as much of the supernatant liquid as possible through a filter and transfer the precipitate to the filter in a stream of water. Wash with suction with 10-ml. portions of water. Carefully add 5-ml. portions of 3:5 sulfuric acid heated to 60° to the filter, gently triturate with a glass rod, and apply suction. Repeat approximately 4 times. Cool to room temperature and dilute to 50 ml. with 3:5 sulfuric acid. Develop with 2,7-naphthalenediol.

Ice cream, cream puffs, mayonnaise. Mix a 5-gram sample with 30 ml. of a 2:5 mixture of hydrochloric acid and methanol. Repeatedly wash the carboxymethylcellulose precipitate with the hydrochloric acid-methanol mixture. Extract the precipitate with 30 ml. of 10% sodium hydroxide solution for 30 minutes with occasional shaking. Filter with suction and dilute the filtrate to 250 ml.[72] Develop a 1-ml. aliquot containing 0.004-0.02 mg. of carboxymethylcellulose with anthrone.

Procedure—*By 2,7-naphthalenediol.* To a 2-ml. aliquot containing 0.02-0.3 mg. of carboxymethylcellulose add 20 ml. of 0.05% 2,7-naphthalenediol reagent solution in concentrated sulfuric acid which has been allowed to stand at least 18 hours in the dark before use. Stopper and shake carefully. Heat at 100° for 3 hours. Cool in ice for 5 minutes and add 20 ml. of 3:5 sulfuric acid. Mix thoroughly and let stand for 30 minutes. Read at 530 mμ against a reagent blank.

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[71] C. R. Szalkowski and W. J. Mader, *J. Am. Pharm. Assoc.* **44**, 533-5 (1955); Cf. E. Wurz, *Österr. Chem.-Ztg.* **56**, 285-6 (1955).

[72] Y. Sakagami and Y. Shiraishi, *Bull. Inst. Public Health (Tokyo)* **4**, 7-9 (1955).

HYDROXAMIC ACIDS

The hydroxamic acid is converted to ferric formohydroxamate and read at 515 mμ,[73] a reaction frequently used for determination of other structures by conversion to hydroxamic acids. As an example see page 289.

Procedure—A suitable sample consists of a 0.5-1 ml. of aqueous or alcoholic solution of hydroxamic acid or alkaline hydroxamate containing 0.1-3 mg. per ml. If the sample is in alcoholic solution, evaporate to dryness and dilute to the original volume with water. Add 0.2 ml. of 1:49 sulfuric acid and seal. Heat the closed ampule in an oil bath at 135-40° for 30 minutes, cool, and filter. Treat the hydrolyzed liquid with 0.5 ml. of a neutral 40% formaldehyde solution and 2.5 ml. of 1% ferric ammonium sulfate solution, which has been acidified with 1 ml. of 1:49 sulfuric acid. If the solution becomes turbid, add 1-2 ml. of ethanol. Dilute to 10 ml. with water and add 50-100 mg. of ammonium persulfate. Read after 10 minutes at 515 mμ.

SALICYLOHYDROXAMIC ACID

Salicylohydroxamic acid or its bromo derivative in the range of 0.01-0.02 mg. can be determined by the violet color formed by reaction with vanadic acid.[74]

Procedure—As a reagent, dissolve 0.25 gram of sodium or ammonium vanadate in 20 ml. of 1:35 sulfuric acid and dilute to 100 ml. Add 1 ml. of reagent and 2 ml. of water to a 2-ml. sample containing 0.01-0.02 mg. of salicylohydroxamic acid. Read the violet color at 610 mμ.

N-1-NAPHTHYLPHTHALAMIC ACID

Hydrolysis of the amide linkage of *N*-1-naphthylphthalamic acid gives 1-naphthylamine and sodium phthalate, the former of which is steam distilled from basic solution to separate it from other substances likely to interfere, and is then coupled with diazotized sulfanilic acid to give an azo dye.[75] The hydrolysis and steam distillation occur simultaneously. The diazonium reagent is stable for several minutes. An excess of sulfanilic acid is used in preparing the reagent because otherwise the red dye formed is not stable. Beer's law is followed.

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[73] E. E. Vonesch and O. A. Guagnini, *Anales asoc. quím. Argentina* **43,** 101-5 (1955).
[74] J. Venulet, *Bull. acad. polon. sci. III,* **2,** 195-8 (1954).

Sample—Sample sizes depend on the nature of the sample and the amount of 1-naphthylamine suspected. However, foaming difficulties encountered with sweet potatoes, cottonseed, and lima beans limit the size of these samples to 5 grams each. Cucumbers, summer and winter squash require 30-gram samples. To obtain a representative sample, chop, mix, or blend a pound or more of the sample to give a subsample of 250 ml.

Procedure—*By diazotized sulfanilic acid.* Wash a sample containing not more than 0.02 mg. of N-1-naphthylphthalamic acid into the hydrolysis flask shown in Figure 17 with 30 ml. of 30% sodium hydroxide solution. Swirl to mix, connect the distillation apparatus, and heat for approximately 20 minutes or until 15 ml. of distillate are collected.

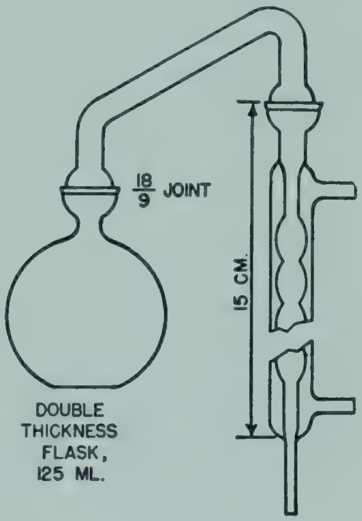

FIG. 17

Apparatus for hydrolysis to and steam distillation of 1-naphthylamine

To prepare the diazonium reagent, dissolve 1 gram of recrystallized sulfanilic acid in 100 ml. of 30% acetic acid by warming. Prepare a 0.12% sodium nitrite solution. Mix equal volumes of the sulfanilic acid solution and the sodium nitrate solution and let stand 2 to 5 minutes before use. Add 5 ml. of glacial acetic acid to the distillate followed by 10 drops of diazonium reagent. Dilute to 25 ml., let stand for 30 minutes, and read at 534 mμ against a reagent blank. For samples of high-residue type of unknown quantities of amine, collect an additional 5 ml. of distillate and test for the presence of 1-naphthylamine by adding acetic acid and the diazonium reagent.

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[75] A. E. Smith and G. M. Stone, *Anal. Chem.* 25, 1397-9 (1953).

CAMPHOCARBOXYLIC ACID

The color of camphocarboxylic acid with 2,4-dinitrophenylhydrazine is read in alkaline solution.[76]

Procedure—Dilute the sample of camphocarboxylic acid in alkaline solution with 0.32% sodium hydroxide solution to about 1.5 mg. per ml. Mix 1 ml. with 1 ml. of 0.15% 2,4-dinitrophenylhydrazine in concentrated sulfuric acid. Heat for 2 hours at 100° and cool. Add 1.4% potassium hydroxide in several portions and finally dilute to 100 ml. with the same solvent. Filter after 10 minutes. Read after 1 hour at 635 or 534 mμ against a reagent blank.

KYNURENIC ACID, XANTHURENIC ACID

Xanthurenic acid is measured by its fluorescence in strong alkali and kynurenic acid is measured by its fluorescence in strong sulfuric acid. The acids do not interfere with each other.[77] The fluorometric method has the advantage over the spectrophotometric method in that both acids may be measured in the same effluent. The phosphate buffer may be eliminated, although it contributes slightly to the stability of the fluorescence. Beer's law is followed. Quinoline derivatives such as quinaldic acid, 8-methyl ether of xanthurenic acid, and 8-hydroxyquinaldic acid do not interfere.

Sample—*Urine*. Suspend 200-400 mesh Dowex 50 containing 12% cross-linkage in water and remove the very fine and very coarse particles. Wash 1500 ml. of the packed resin with 25 liters of 2:1 hydrochloric acid, 12 liters of 1:2 hydrochloric acid, and 10 liters of water at a flow rate of 750 ml. per hour.[78] Prepare an ion-exchange column 1.2 cm. × 15 cm. attached to the bottom of a 250-ml. flask. Place glass wool above a constriction near the bottom of the tube and add 3 cm. of Dowex 50. Wash the column with 50 ml. of 1:2 hydrochloric acid and 100 ml. of water. Dilute each of two samples to 120 ml. with water. To one sample add 1 ml. of kynurenic acid standard solution and 1 ml. of standard xanthurenic acid solution. To both samples add 30 ml. of 1:110 hydrochloric acid and mix. Add the solutions to Dowex columns and wash the columns with 50 ml. of 1:59 hydrochloric acid, 100 ml. of 1:24 hydrochloric acid, and

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[76] Enzo Maggiorelli, *Boll. chim. farm.* **98, 323-6 (1959).**

[77] K. Satoh and J. M. Price, *J. Biol. Chem.* **230, 781-9 (1958).**

[78] J. M. Price, *Ibid.* **211, 117-24 (1954).**

20 ml. of water. Elute the kynurenic and xanthurenic acids with 396 ml. of water, carrying out the entire procedure under gravity flow rates. Add 4 ml. of a 0.5 *M* phosphate buffer at pH 7.4 (Vol. I, p. 174) to dilute the eluate to 400 ml.

Procedure—*Kynurenic acid.* Dilute a 1 to 6 ml. aliquot of the effluent to 6 ml. with 0.005 *M* phosphate buffer at pH 7.4. Slowly add 4 ml. of concentrated sulfuric acid, mix, and cool rapidly. Read in 1 hour with an activating wave length of 340 mμ and a fluorescence wave length of 435 mμ.

Xanthurenic acid. Dilute 0.1 to 5 ml. of the effluent to 5 ml. with 0.005 *M* phosphate buffer at pH 7.4 and add 5 ml. of a saturated sodium hydroxide solution. Mix and centrifuge for 5 minutes. Read after 1 hour at 530 mμ by activating at 370 mμ.

Gibberellic and Gibberellenic Acids

Gibberellic acid is a colorless, polycyclic, unsaturated, monobasic acid containing a lactone group. It has a molecular weight of 346 and only end absorption in the ultraviolet region. But it produces a wine-colored solution with a blue fluorescence when treated with concentrated sulfuric acid.[79] The most important decomposition product of gibberellic acid is gibberellenic acid. Since the fluorometric method does not distinguish between gibberellic acid and gibberellenic acid the total measured must be corrected for the amount of gibberellenic acid in the sample by reading the gibberellenic acid at 254 mμ after extraction with *n*-butanol followed by extraction with phosphate buffer at pH 7.5. Alternatively, the gibberellic acid may be separated from the gibberellenic acid by chromatography on a potassium bicarbonate column.

In the fluorescence procedure, the cold sulfuric acid must be added to the cold sample in the same manner for all tests and the flasks agitated the same as variations causes variations in fluorescence. In the determination of gibberellenic acid at 254 mμ, gibberellic acid is converted to gibberellenic acid under the conditions of the method. However, the conversion is slow enough not to interfere. Beer's law is followed for 0.005-0.02 mg. gibberellic acid per ml.

Procedure—*Fermentation broths.* Mix the whole broth from the fermenter with an equal volume of methanol and filter. If the methanol does not cause precipitation, dilute with water or methanol. If the concentration is low or the solvent from the fermentation is likely to interfere, evaporate a small measured volume to dryness, dissolve the residue in 5 ml. of warm methanol per gram of gibberellic acid, and dilute with methanol or water so that the gibberellic acid concentration is 0.02 mg. per ml.

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[79] F. Kavanagh and N. R. Kuzel, *J. Agr. Food Chem.* 6, 459-63 (1958).

Separation of gibberellic acid from gibberellenic acid. Prepare a mixture of 50 grams of granular potassium bicarbonate and 20 grams of anhydrous sodium sulfate. Add to a 20 × 250-mm. chromatography column fitted with a stopcock and a 6-mm. exit opening. Add the salts in small quantities with gentle tapping to ensure uniform packing. Add 5 ml. of a sample solution containing 2-3 mg. of gibberellic acid and let stand for 10 minutes. Add 10 grams of potassium bicarbonate and top with a small glass wool plug. Elute the column at the rate of 3 ml. per minute with 1% acetonitrile solution and collect a total of 200 m. Determine the amount of gibberellic acid in 1 ml. of the effluent by the fluorometric method. Elute gibberellenic acid with methanol.

Gibberellic acid by fluorescence. Chill a sample containing 0.005-0.02 mg. of gibberellic acid per ml. in water, methanol, or acetonitrile. Add 25 ml. of chilled 6:1 sulfuric acid with continuous shaking, allowing the acid to flow in as rapidly as possible. Let stand for at least 1 hour before reading. Correct for gibberellenic acid if present.

Gibberellenic acid. Mix the whole broth with an equal volume of methanol and filter. If the sample contains a ketone or other interfering solvent, evaporate a measured portion of the sample to dryness and dissolve the residue in methanol. To a 1-ml. clear sample containing less than 0.2 mg. of gibberellenic acid, add 5 ml. of acid buffer at pH 1.1 prepared by diluting 50 grams of sodium chloride and 8 ml. of concentrated hydrochloric acid to 1 liter. Add 10 ml. of *n*-butanol, shake, and allow the layers to separate, centrifuging if necessary. Discard the lower aqueous layer. Add 10 ml. of phosphate buffer at pH 7.5 prepared by diluting a solution of 20 grams of monobasic potassium phosphate and 2.5 grams of dibasic potassium phosphate to 1 liter and saturating with *n*-butanol. Shake and allow the layers to separate. To the lower layer add 1 ml. of methanol to prevent the separation of butanol which causes turbidity when warmed. Read at 254 mμ against a mixture of 10 ml. of the phosphate buffer and 1 ml. of methanol.

WARFARIN

Warfarin is read directly in rodenticides in sodium pyrophosphate solution after extraction with ether. For samples which are difficult to extract with ether, an Attapulgus clay column is used to remove impurities.[80]

Procedure—*Rodenticides containing 0.5% warfarin.* Shake a 0.6-gram sample with 50 ml. of ether for 30 minutes. Centrifuge until clear. Add 2 ml. of the supernatant liquid to 10 ml. of 1% sodium pyrophosphate solution and shake vigorously for 2 minutes. Centrifuge until the lower aqueous layer is clear. Draw off the upper ether layer. Add 2 ml. of ether to the aqueous layer, shake, and centrifuge. Draw off the ether phase and any emulsion formed at the interface. If the aqueous layer is turbid or colored, repeat the ether extraction. Extract the aqueous layer with petroleum ether previously purified by shaking with 1% sodium pyrophosphate solution and dried over anhydrous sodium sulfate. Remove the petroleum ether phase and draw air over the surface of the aqueous layer to evaporate any remaining solvent. Read at 308 mμ. To calculate % warfarin multiply absorbance at 308 mμ by 0.9.

Pelleted baits difficult to extract with ether. Shake a 2-gram sample with 50 ml. of 1% sodium pyrophosphate solution for 1 hour. Centrifuge for 5 minutes. Shake 25 ml. of the supernatant liquid with 5 ml. of 1:4 hydrochloric acid and 50 ml. of a 4:1 mixture of hexane and ether for 10 minutes. Evaporate 20 ml. of the ether phase to dryness at 100° using a gentle stream of air. Take up the residue with three 1-ml. portions of ether and pass the solution through a column containing 2 grams of Attapulgus clay. Collect 20-25 ml. of ether and shake with 10 ml. of 1% sodium pyrophosphate solution. Remove ether and wash the pyrophosphate phase with the ether-hexane mixture. Read at 308 mμ.

ISOHUMULONES

The isohumulones from beer are developed with phosphotungstic-phosphomolybdic acid.[81]

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[80] J. B. LaClair, *J. Assoc. Offic. Agr. Chemists* 37, 634-8 (1954).

[81] F. L. Rigby and J. L. Bethune, *Am. Soc. Brewing Chemists, Proc.* **1953,** 119-29; R. D. Hall, *Proc. Eur. Brew. Conv. Copenhagen,* **1955,** 314; W. J. Klopper, *Brewers Digest* 30, 63-4, 66 (1955); J. L. Orvades, J. Jacovac, and M. W. Brenner, *American Brewer,* **92,** No. 2, 42-3 (1959).

Procedure—Mix 20 ml. of decarbonated beer, 2 ml. of 1:3 hydrochloric acid, and 40 ml. of isooctane. Shake vigorously for 30 seconds and separate the layers, discarding the lower layer. Shake 25 ml. of the isooctane layer with 25 ml. of 1.16% sodium carbonate solution.

As reagent mix 750 ml. of water, 100 grams of sodium tungstate dihydrate, 20 grams of phosphomolybdic acid, and 50 ml. of 85% phosphoric acid. Reflux for 2 hours, cool, and dilute to 1 liter.

Mix 20 ml. of the aqueous layer of the sample with 0.75 ml. of reagent and add 2.5 ml. of saturated sodium carbonate solution. Mix and dilute to 25 ml. After 1 hour filter if necessary. Read against a reagent blank at 735 mμ. The ppm. of isohumulones = 140 × optical density − 2.

CHAPTER 18

SULFUR DERIVATIVES

NECESSARILY some sulfur derivatives also have other active groups. Some occur in other chapters. Thus thioglycolic acid which is a substituted aliphatic acid is so classified, page 318. Similarly the sulfonamides appear in Vol. IV, Chapter 5.

Cysteine may be oxidized to cystine and determined. One method given for cystine determines both. Diphenylthiourea reacts with potassium mercuric iodide in aqueous ethanol to give a yellow complex.[1]

For determination of thiotoluenes in toluene the alloxan method described for thiophene is preferable to the isatin method.[2] 6-Mercaptopurine and its by-product hypoxanthine are read directly. The first is read at 325 mμ. The second is determined by the ratio of the absorption at 253/325 mμ.[3] When allicin, the antibacterial in garlic, is decomposed by enzymes it forms pyruvic acid. The latter is then determined as the 2,4-dinitrophenylhydrazone (page 337) in alkaline solution.[4]

The fluorescence of thiopental at pH 13 when activated at 315 mμ shows a peak at 530 mμ. Similarly thiamylal (Surital) at pH 13 activated at 310 mμ has a peak at 530 mμ.[5] For determination, N^1-sulfanilyl-N^2-butylcarbamide is diazotized and coupled with thiocol.[6] The resulting absorption is read at 255 mμ. Both 4-methyl-2-thiouracil and 4-propyl-2-thiouracil are determined with sodium nitroprusside as described for 2-thiouracil. Dibenzothioxin, phenoxathiin, has maxima at 237 mμ and 294 mμ.[7] Ethanolic solutions are appropriate.

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[1] Francisco Hernández Gutiérrez, *Anales real soc. españ. fís. y quím.* (*Madrid*) **53B,** 211-6 (1957).

[2] J. Croisier, M. Hoestlandt, and A. Mennessier, *Compt. rend. congr. ind. gaz., 68th Congr. Paris,* **1951,** 914-7.

[3] T. Noto, H. Sawada, Y. Sato, N. Fukuda, and Y. Inoue, *Tanabe Seiyaku Kenkyu Nempo* **3,** 53-8 (1958).

[4] Herbert Jäger, *Arch. Pharm.* **288,** 145-8 (1955).

[5] S. Udenfriend, D. E. Duggan, B. M. Vasta, and B. B. Brodie, *J. Pharmacol. Exptl. Therap.* **120,** 26-32 (1957).

[6] A. Häussler, *Arzneimittel-Forsch.* **6,** 393-4 (1956).

[7] F. Capitán-García and R. Parellada-Bellod, *Anales real soc. espan. fís. y quím.* (*Madrid*) **53B,** 205-10 (1957).

Lactargol is precipitated by tripotassium hexathiocyano chromate. This precipitate is read in acetone at 574 mμ.[8]

SULFHYDRYL GROUPS

The familiar reaction of mercury and dithizone (Vol. IIA, p. 36) has been adapted to a difference method for sulfhydryls. The sulfhydryl is first reacted with a known excess of *p*-mercuribenzoate. The unreacted *p*-mercuribenzoate is then determined by its bleaching effect on dithizone.[9] For many biological samples this is advantageous as avoiding interfering absorption, particularly in the ultraviolet. Thus it is appropriate for following the rate of reaction of enzymes with *p*-chloromercuribenzoate.

When present in excess, *N*-ethylmaleimide reacts stoichiometrically with sulfhydryl groups and the decrease in absorption at 300 mμ is used as a quantitative method.[10]

The *N*-ethylmaleimide concentration must be in at least 10% excess of the sulfhydryl concentration. Interference of purines, pyrimidines, nucleosides, and nucleotides is negligible at 300 mμ and pH 6. If needed, wave lengths up to 320 mμ can be used without great loss in sensitivity. The reaction is highly specific for sulfhydryl groups, although thiolhistidine and ergothioneine do not react.

Protein-bound, water-insoluble sulfhydryl groups form a purple color with tetrazotized di-*o*-anisidine.[11] The color is stable for several hours.

The *S*-nitroso derivatives of thiols are easily hydrolyzed in the presence of the mercuric ion. The liberated nitrous acid reacts with sulfanilamide and *N*-1-naphthylethylenediamine to form a diazonium salt.[12] This method is applicable to the determination of about 0.1 mg. of cysteine without interference from large excesses of amino acids in protein hydrolysates. Dialkylamines do not interfere.

Bis(*p*-nitrophenyl)disulfide reacts quantitatively with mercaptans to release 1 mole of *p*-nitrobenzenethiol per mole of mercaptan which is read at 412 mμ.[13] The reaction is specific for compounds containing a sulfur atom capable of existing as an anion at pH 8 such as cysteine, dithiobiuret, dodecylmercaptan, glutathione, 2-aminoethanethiol, 2-mercaptoethanol, sodium sulfide, sodium sulfite, sodium thiocyanate, sodium thiosulfate, thioacetamide, thiopental, and thiourea. The following compounds will not form color with the reagent: methionine, ethionine, tetrahydro-1-methylthiophenium iodide, 2,2′-hexamethylenebis(2-thiopseudourea)dihydrochloride, and 6,6′-thiodi-2-naphthol. The yellow color develops in less than 10 seconds, with the exception of whole blood samples which require 1 hour, and fades after 30 minutes. Heavy metal ions such as mercury, silver, and lead interfere. Lowering of the pH causes the color to fade.

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[8] P. Spacu, E. Antonescu, and C. Gheorghin, *Acad. rep. populare Romîne, Studii cercetări chim.* **6**, 573-84 (1958).

[9] I. Fridovich and P. Handler, *Anal. Chem.* **29**, 1219-20 (1957).

[10] E. Friedmann, D. H. Marrian, and I. Simon-Ruess, *Brit. J. Pharmacol.* **4**, 105-8 (1949); E. Friedmann, *Biochem. et Biophys. Acta* **9**, 65-75 (1952); J. D. Gregory, *J. Am. Chem. Soc.* **77**, 3922-3 (1955); J. Broekhuysen, *Anal. Chim. Acta* **19**, 542-7 (1958); Nicholas M. Alexander, *Anal. Chem.* **30**, 1292-4 (1958); E. Roberts and G. Rouser, *Ibid.* **30**, 1291-2 (1958).

[11] P. Flesch, S. Golomg, and A. Satanove, *J. Lab. Clin. Med.* **43**, 957-9 (1954).

[12] B. Saville, *Analyst* **83**, 670-2 (1958).

A general reaction for mercaptans is that with nitrous acid as given here for 2-hydroxyethanethiol.[14] (Cf. page 318.)

Procedure—*By* p-*chloromercuribenzoate.* To prepare the reagent, dissolve 16.2 mg. of *p*-chloromercuribenzoate in water containing a few drops of alkali. Add 10 ml. of *M* potassium phosphate buffer for pH 7 (Cf. Vol. I, p. 174) and 5 ml. of 8.7% potassium sulfate solution. Dilute to 100 ml. and store in the cold. As dithizone reagent dissolve 4 mg. of diphenylthiocarbazone in 100 ml. of carbon tetrachloride. Filter and use promptly. The absorbance should be approximately 0.84 at 625 mμ. To 4 ml. of the dithizone reagent, add 1-10 ml. of reagent containing 0.002-0.02% of *p*-chloromercuribenzoate. Shake vigorously for 1 minute, separate, and read the organic phase at 625 mμ.

Sulfhydryls. This is exemplified by but not limited to cysteine. To 0.15 ml. of *p*-chloromercuribenzoate reagent add 1 to 5 ml. of unknown containing 0.005-0.05 micromole of sulfhydryl in neutral or weakly acidic solution. Mix and add 4 ml. of dithizone reagent. Shake vigorously for 1 minute and read the organic phase at 625 mμ. The decrease from a corresponding result with the reagent alone measures the sulfhydryl.

Protein sulfhydryl. To 0.7 ml. of *p*-chloromercuribenzoate reagent, add 0.5 ml. of *M* potassium phosphate buffer for pH 7, 0.5 ml. of 8.7% potassium sulfate solution, and 1.1 ml. of water. At zero time add 0.7 ml. of protein solution containing about 0.25 micromole of sulfhydryl. Add

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[13] George L. Ellman, *Arch. Biochem. and Biophys.* **74**, 443-50 (1958).

[14] F. N. Woodward, *Analyst* **74**, 179-82 (1948); G. T. Walker and F. M. Freeman, *Mfg. Chemists* **26**, 11-3 (1955).

0.5 ml. of this mixture to 4 ml. of dithizone reagent, shake vigorously, and read at 625 mμ.

By N-*ethylmaleimide.* Read the absorbance of a solution in 0.1 *M* phosphate buffer at pH 6.8 with 0.015% *N*-ethylmaleimide and a sulfhydryl concentration between 0.0001 *M* and 0.0009 *M* at 300 mμ. Also read the absorbance of the *N*-ethylmaleimide solution in the phosphate buffer. Divide the difference in absorption between the reacted and unreacted *N*-ethylmaleimide by the molar extinction coefficient of the compound to determine the molar sulfhydryl concentration of the sample.

*By di-*o-*anisidine. Protein.* As a reagent dissolve 1 mg. of 2,2′-dihydroxy-6,6′-dinaphthyl disulfide in 4 ml. of absolute ethanol and dilute to 10 ml. with 0.1 *M* barbiturate buffer at pH 8.5. To 2 ml. of reagent add 5-15 mg. of dried protein. Incubate at 50° for 1 hour. Centrifuge and add 0.2 ml. of the supernatant liquid to 0.5 ml. of 10% sodium dodecyl sulfate solution. Add 0.5 ml. of 0.05% solution of tetrazotized di-*o*-anisidine and dilute to 10 ml. with water. Read at 560 mμ.

As the diazonium salt by N-*1-naphthylenediamine.* To 5 ml. of a mixture of 1 volume of 0.07% sodium nitrite solution and 9 volumes of 1:35 sulfuric acid, add 1 ml. of a thiol in a concentration equivalent to 0.025-0.6 mg. of cysteine in water or aqueous ethanol. Let stand for 30 seconds to 5 minutes to form the *S*-nitroso derivative and add 1 ml. of 0.5% ammonium sulfamate solution. Stopper and shake well for a few seconds to ensure complete removal of excess nitrous acid. After 1-2 minutes, rapidly add 10 ml. of a 1:4 mixture of 1% mercuric chloride solution and 3.4% sulfanilamide solution in 1:29 hydrochloric acid to hydrolyze the *S*-nitrosothiol and form the diazonium salt. Dilute to 25 ml. with 0.1% *N*-1-naphthylethylenediamine dihydrochloride solution in 1:29 hydrochloric acid. Read after 10 minutes at 550 mμ.

By bis(p-*nitrophenyl*)*disulfide. Serum.* Mix 1 ml. of 0.1 *M* phosphate buffer at pH 8, 5.9 ml. of water, 2.7 ml. of acetone, and 0.3 ml. of 0.0308% bis(*p*-nitrophenyl)disulfide solution in acetone. Add 0.1 ml. of serum and read immediately at 412 mμ against a blank containing all the reagents with the exception of bis(*p*-nitrophenyl)disulfide and containing 0.3 ml. of additional acetone.

Whole blood. Mix 5 ml. of water with 0.01 ml. of blood sample. Add 2 ml. of 0.1 *M* phosphate buffer at pH 8 and 2.7 ml. of acetone. Let stand at room temperature for 1 hour. Add 0.3 ml. of 0.0308% bis(*p*-nitrophenyl) disulfide solution in acetone and read at 412 mμ against a blank containing all the reagent except bis(*p*-nitrophenyl)disulfide and containing 0.3 ml. of additional acetone.

General. To a 4-ml. neutral, relatively salt-free sample add 1 ml. of 0.1 *M* phosphate buffer at pH 8, 4 ml. of acetone, and 1 ml. of 0.0308% bis(*p*-nitrophenyl)disulfide solution in acetone. Read at 412 mμ against a blank.

By nitrous acid. 2-Hydroxyethanediol. Dilute a sample containing 0.1-0.4 gram of a thiol, such as 2-hydroxyethanethiol, to 50 ml. with 95% ethanol. To a 6-ml. aliquot in a cuvet add 1 ml. of 27.6% sodium nitrite solution and 2 ml. of glacial acetic acid. Mix. Read exactly 3 minutes later.

CARBONYL SULFIDE

Carbonyl sulfide is hydrolyzed in dilute base to the sulfide ion determined as methylene blue.[15] The method was developed for analysis of synthesis gas. Potassium hydroxide is preferred over sodium hydroxide as the absorbant since concentrations of sodium hydroxide of the order of 0.04% are affected by carbon dioxide and higher concentrations gave poor absorption. Carbon disulfide, ethyl mercaptan, and thiophene do not interfere. Hydrogen sulfide is removed by passing over iron oxide. Large amounts of carbon dioxide are absorbed in 40% potassium hydroxide solution which does not absorb carbonyl sulfide.

Carbonyl sulfide is extracted from petroleum refinery gases as piperidine oxythiocarbamate by an aqueous solution of piperidine.[16] The complex exhibits a strong absorption in the ultraviolet. The color is stable for 18 hours and Beer's law is followed. Hydrogen, oxygen, nitrogen, carbon monoxide, methane, ethane, acetylene, ethylene, propane, and propylene do not interfere. Interferences of hydrogen sulfide and methyl mercaptan are eliminated by scrubbing the gas with a mixture of sodium carbonate and cadmium chloride followed by 30% sodium hydroxide. The sodium hydroxide is necessary to remove carbon dioxide released by the hydrogen sulfide and also removes sulfur dioxide and other acidic components of the gas. If care is not taken, large quantities of carbonyl sulfide may be lost by hydrolysis with sodium hydroxide. Some carbonyl sulfide is lost even with standardized flow rates, type of scrubber, and height of scrubbing liquid.

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[15] L. A. Pursglove and H. W. Wainwright, *Anal. Chem.* **26**, 1835-9 (1954).

[16] R. E. Snyder and R. O. Clark, *Ibid.* **27**, 1167-71 (1955); J. O'Hara, W. M. Keely, and H. W. Fleming, *Ibid.* **28**, 466-70 (1956).

Approximately 10% of the carbonyl sulfide hydrolyzes when the gas flow rate is 25 liters per hour using 80 ml. of 30% sodium hydroxide solution in the scrubber described. This results in 3.5 inches of reagent above the frit end. It may be necessary to add 10 drops of an antifoaming agent to reduce foaming. The hydrolysis may also be decreased by increasing the flow rate. The interferences of carbon disulfide, thiophene, and 1,3-butadiene are canceled out by subtracting a second piperidine extract from the first. A lowering of pH causes the formation of the piperidinium solution at wave lengths below 240 mμ, the decrease continuing until ionization is complete. Addition of alkali results in an increase in absorption of the piperidine solution. An accuracy of 1 ppm. of carbonyl sulfide in the range of 1-50 ppm. is obtained.

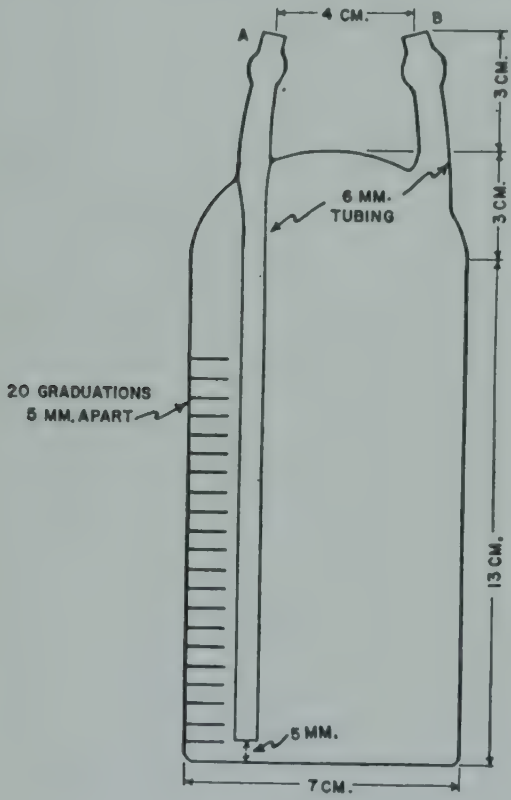

FIG. 18

Graduated absorption bottle for determination of carbonyl sulfide

Procedure—*As methylene blue.* The absorption bottle is shown in Figure 18. Fill this with 0.56% potassium hydroxide solution from a leveling bottle connected at point A. Introduce the gas sample through point B by lowering the leveling bottle. Continue until a little more than 50 ml. of absorbant is left in the bottle. Close the tubes to the openings at A and B. Open point B to bring the contents of the absorption bottle to atmospheric pressure and close again. Connect the tubing from point B to A. Shake the bottle thoroughly for 20 minutes, pour out the absorbant, and measure the volume.

As the reagent dissolve 0.15 gram of *p*-aminodimethylaniline in 150 ml. of 2:1 sulfuric acid. Cool a 50-ml. portion of absorbant solution to not over 10°. Add 5 ml. of the reagent and mix. Add 1 ml. of 2.7% solution of ferric chloride hexahydrate in 1:1 hydrochloric acid. Mix and read after 15-30 minutes when the maximum blue color will have developed.

Calculate carbonyl sulfide as

$$C_g = C_s\,(V_s/50)(28,\,300)\;(100/V_g c_f)$$

C_g = the concentration of carbonyl sulfide in the gas stream in grains of sulfur per 100 standard cubic feet

C_s = the concentration of sulfide sulfur in the solution in grains of sulfur per 50 ml. as taken from the methylene blue calibration curve

V_s = the total volume of absorbing solution in ml.

V_g = the volume of gas sample in ml. obtained by subtracting V_s from the total volume of the absorbing bottle

c_f = a correction factor from cubic feet to standard cubic feet.

Alternatively, connect the absorption bottle and allow it to be purged by a volume of gas equal to 5-6 times the volume of the bottle. Close both openings A and B. Remove the clamp from A and drain 50 ml. of 0.56% potassium hydroxide solution into the bottle by gravity, releasing

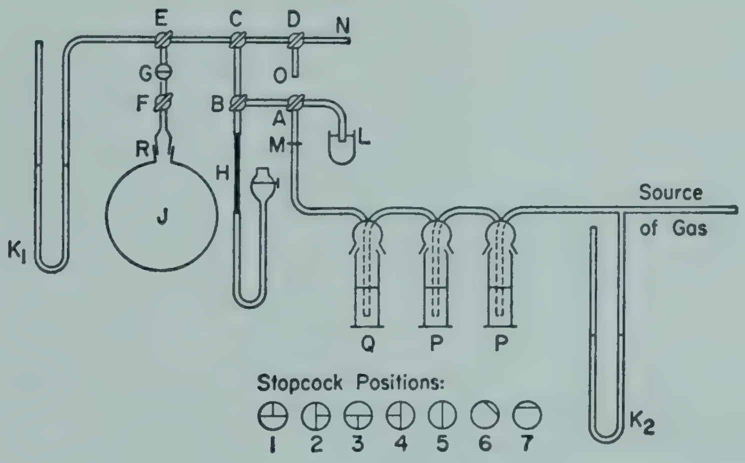

FIG. 19

Apparatus for determination of carbonyl sulfide

A, C, D, E, T stopcocks; B, 120° stopcock; F, Straight-bore stopcock; G, 12/5 ball joint; H, 1.00-ml. buret; I, Mercury reservoir; J, Calibrated 5-liter sampling flask; K_1, K_2, Mercury manometers; L, Dip tube; M, Gas sample inlet to manifold; N, Vacuum line; O, Vent; P, Shaw's reagent scrubbers; Q, 30% sodium hydroxide scrubber; R, 45/50 standard-taper joint.

the clamp at B to equalize the pressure when necessary. Follow the procedure above starting at "Connect the tubing from point B to point A." Calculate carbonyl sulfide as follows:

$$C_g = C_s(28{,}000)(100/V_g c_f)$$

in which

$$V_g = \text{the bottle volume minus 50 ml.}$$

As piperidine oxythiocarbamate. The apparatus is shown in Figure 19. As a reagent, dissolve 1 ml. of piperidine in 2 liters of water and store under nitrogen to prevent reaction with carbon dioxide in the atmosphere. To prepare the solution for the scrubbers *P*, mix 1 volume of 5.3% sodium carbonate solution and 7 volumes of 10% camium chloride solution.

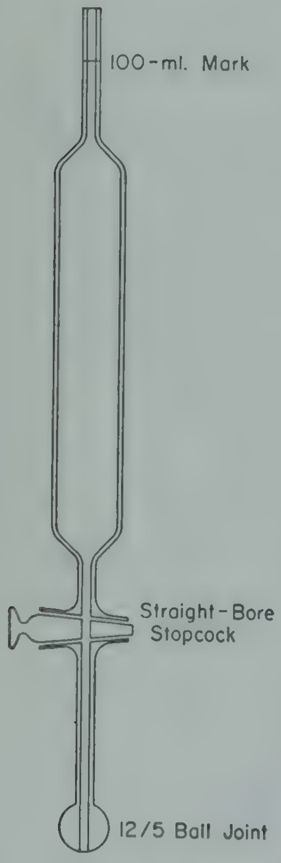

FIG. 20

Special 100-ml. pipet for determination of carbonyl sulfide

Pass the petroleum refinery gas through the gas sampling apparatus at a rate of 25 liters per hour. Evacuate the calibrated flask, J, and the various lines down to B, as indicated by the mercury manometers K_1 and K_2 as follows. Place D in position 1, C and E in position 3, B in position 7, and F in position 5. With A in position 3, B in position 6, C in position 2, and D in position 4, slowly pass the gas around the A-B-C-D part of the manifold with a slight flow being maintained through the dip tube L. After sufficient flushing, turn C to position 4, E to position 3, and F to position 5. Slowly fill the evacuated flask J with gas as indicated by the mercury manometer K_1 by regulating F while preserving a constant flow through the dip tube L.

After the calibration flask J is filled with gas, disconnect it from the manifold at G. Rinse the special pipet, Figure 20, with several portions of the piperidine solution and fill to 100 ml. Apply a small amount of pressure to transfer the extraction solution from the pipet to the calibrated flask J. Mix the calibrated flask J vigorously in a mechanical shaker for 80 minutes. Read a portion at 230 mμ against piperidine. Remove the solution remaining in the flask, rinse the flask with 100-ml. portions of fresh piperidine solution and pressure a final 100-ml. portion of piperidine solution into the flask as above starting with "Rinse the special pipet . . ." and ending with "Read a portion at 230 mμ against piperidine solution."

Calculate carbonyl sulfide as follows:

$$\text{carbonyl sulfide in ppm.} = \frac{25{,}600\,(G_1 - G_2)}{V}$$

where

G_1 = grains of carbonyl sulfide per 100 ml. of aqueous piperidine solution obtained from a calibration curve corresponding to the absorbance value of the extraction solution at 230 mμ after the first 80-minute period of contact

G_2 = the same as G_1 except the absorbance value after the second 80-minute shaking is taken

V = the volume of hydrocarbon refinery gas in liters at 60°F. and 1 atmosphere.

THIOUREA

Thiourea and nitrite react to form thiocyanic acid which is measured as the familiar iron thiocyanate complex. When an excess of nitrite is used, the thiocyanic acid produced is proportional to the thiourea concentration.[17] The pH is maintained between 3 and 5 by an acetic acid-acetate buffer. Aluminum, ammonium, barium, calcium, cobalt, lead, lithium, magnesium, manganese, potassium, sodium, strontium, uranyl, zinc, chloride, bromide, nitrate, sulfate, and tungstate ions can be tolerated in concentration of 500 ppm. The following ions interfere in the concentration in ppm. given: chromium 100, nickel 150, cadmium 60, vanadate 85, chromate 25, carbonate 15, arsenate 15, phosphate 30, iodide 5, and fluoride 60. A precipitate forms when 0.5 mg. of the following ions are present: copper, tin, mercury, bismuth, ferric, silver, perchlorate, dichromate, thiosulfate, permanganate, and sulfide. A cation-exchange resin converted to the sodium salt form removes 25 mg. of ferric and cupric ion from solutions containing 0.475 mg. of nitrite.

A hydrated cyanoferrate reagent, prepared from sodium nitroprusside and hydroxylamine hydrochloride, is used to develop thiourea.[18] Samples are clarified by trichloroacetic acid and Lloyd's reagent.[19]

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[17] K. Hutchinson and D. F. Boltz, *Ibid.* 30, 54-6 (1958).

[18] "Official Methods of Analysis of the Association of Official Agricultural Chemists," 8th ed., p. 510. Association of Official Agricultural Chemists, Washington, D. C. (1955).

[19] W. O. Winkler, *J. Assoc. Offic. Agr. Chemists,* 39, 662-3 (1956).

Sample—*Orange juice or brined lemon peel.* Blend the sample. If it is too thick, add an equal weight of water. Centrifuge and decant. To each 100 ml. of the supernatant liquid, add 6-8 ml. of 20% trichloroacetic acid solution with mixing. Dilute to 110 ml. with water, add several grams of Lloyd's reagent and shake for 1 minute. Add 1-2 grams of filter aid, mix, and filter with suction. Adjust the pH to 3.5-5.5 with dibasic potassium phosphate. If iron is present, adjust the pH to 7, refilter, and lower the pH to 3.5-5.5 with acetic acid. Develop a 10-ml. portion with hydrated cyanoferrate reagent.

Procedure—*By nitrite and iron.* To prepare the buffer solution, dissolve 3 ml. of glacial acetic acid and 4.9 grams of potassium acetate in water and dilute to 1 liter. To prepare the ferric perchlorate reagent, dissolve 80 grams of hydrated ferric perchlorate and 34 ml. of 75% perchloric acid in water and dilute to 1 liter. Standardize this solution using stannous chloride as the reductant and potassium dichromate as the titratant to be approximately equivalent to 6.534 mg. of iron per ml.

To a solution not exceeding 25 ml. and containing 4-32 ppm. of thiourea, add 5 ml. of 1% potassium nitrite solution and 10 ml. of buffer. Mix, heat at 70°-80° for 30 minutes, and cool. Add 10 ml. of ferric perchlorate reagent. Dilute to 50 ml. with water and read at 455 mμ against a reagent blank.

By hydrated cyanoferrate reagent. Dissolve 0.5 gram of sodium nitroprusside in 10 ml. of water. Mix 0.5 gram of hydroxylamine hydrochloride and 1 gram of sodium bicarbonate and add to the nitroprusside solution. Do not agitate. Let stand until nearly all of the carbon dioxide evolves. Swirl to dissolve any remaining sodium bicarbonate. When evolution of carbon dioxide practically stops, add 0.1 ml. of bromine. A second evolution of gas occurs. When agitation no longer produces gas evolution, dilute to 25 ml. with water and filter. As a reagent, dilute 1 volume with 4 volumes of water.

To a 10-ml. portion of the sample, add 1 ml. of the hydrated cyanoferrate reagent and read at 600-610 mμ after 45 minutes.

ALLYLTHIOHYDANTOIN, GLYCOLYL THIOUREA

Allylthiohydrantoin gives an intense red color with ammonia which is stable for 1 to 2 hours.[20]

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[20] Bjarne Salvesen, *Medd. Norsk. Farm. Selskap* **18**, 107-10 (1956).

Procedure—To a 5-ml. sample in 2-propanol containing 1 mg. of allythiohydantoin add 5 ml. of 25% ammonium hydroxide solution. Read at 523 mμ after 10 minutes.

Aliphatic Sulfides

Aliphatic sulfides and iodine form a 1:1 complex which absorb intensely in the ultraviolet.[21] No other sulfur compound type normally found in the boiling range of gasoline has an appreciable absorption in this region. This differentiates between aliphatic sulfides and aromatic hydrocarbons since the intensity of the aliphatic sulfide complexes is of the order of 1000 times that of those formed with aromatic hydrocarbons. If the aromatic content of the sample and its interference cannot be determined, the method should not be used for samples containing less than 0.05 weight per cent of aliphatic sulfur. Typical compounds so determined are 3-thiopentane and 2-methylthiocyclopentane. Since the absorptivity of different compounds of the class varies somewhat the accuracy is lessened to 4%. No aromatic sulfides boil in the gasoline range.

The sensitivity of the method is increased in analysis of chromatographic fractions by using a large excess of iodine by using a lower sample concentration to decrease the background absorption, and by reading with a photomultiplier attachment.[22] Aromatic and olefinic hydrocarbons present absorb some of the iodine, another reason for needing an excess.

Since lower temperatures favor the complex formation, the analysis should be performed at a specific controlled temperature or a temperature correction applied. The reading should take place immediately as the absorbance changes in time. Carbon tetrachloride is chosen as the solvent in preference to isooctane because of the greater solubility of odine in it.

Procedure—*Crude petroleum oils and their chromatographic fracions.* Dissolve in carbon tetrachloride and dilute so that the solution roduces an absorption falling between 0.4 and 1. A list of appropriate ample weights is as follows:

Prepare an iodine reagent by dissolving 10 grams of iodine in carbon etrachloride and diluting to 1 liter. Dilute a 1-ml. portion to 10 ml. with ne sample solution. Dilute 1 ml. of carbon tetrachloride to 10 ml. with ne sample solution as a blank. Read immediately at 310 mμ. To obtain

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[21] S. H. Hastings, *Anal. Chem.* 25, 420-2 (1953); S. H. Hastings and B. H. Johnson, *id.* 27, 564-5 (1955).

[22] H. V. Drushel and J. F. Miller, *Anal. Chem.* 27, 495-501 (1955).

Aliphatic Sulfide Sulfur in Sample, %	*Approximate Weight of Sample Diluted to 25 Ml. to Obtain Absorbance between 0.4 and 1.0, Mg.*
0.05	60-140
0.10	30-70
0.20	15-35
0.40	8-18
0.60	5-12
0.80	4-9
1.0	3-7
1.5	2-5
2.0	1-3

the blank value for the iodine reagent, dilute 1 ml. of iodine reagent to 10 ml. with carbon tetrachloride and read at 310 mμ against carbon tetrachloride. An alternative blank when sulfur is very low is obtained by removing the sulfide from a portion of the sample with solid mercuric sulfate and washing the residue with 10% aqueous mercuric nitrate solution followed by water.

Calculate aliphatic sulfide sulfur as follows:

$$\text{weight per cent of aliphatic sulfide sulfur} = \frac{(A_1 - A_2) \times 100}{400 \times 0.9 \times c}$$

in which

A_1 is the absorbance of the sample at 310 mμ

A_2 is the absorbance of the iodine blank

c is the concentration in grams per liter of the sample solution.

1,2-Bis(Methylthio)ethane

1,2-Bis(methylthio)ethane coordinates with the cobaltous ion. Ther the complex undergoes oxidation to the cobaltic ion and is measured by its oxidation of the redox indicator *o*-dianisidine.[23] Benzidine and *o*-toli dine may also be used. Since dibutylamine gives an identical spectrun under the same conditions with the same redox indicator, but a differen spectrum in the absence of the redox indicator, it is assumed that th spectrum is characteristic of the redox indicator and not the compounc

Strong oxidizing agents such as hydrogen peroxide, chlorine, or mixture of nitric oxide and nitrogen dioxide must be present. This metho allows the estimation of the dithioether in the presence of the mono ioether or akyl sulfide as the monothioether does not form a complex ith cobaltous chloride. Beer's law is followed for 0.015-0.09 mg. of 2-bis(methylthio)-ethane at 448 mμ.

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[23] J. S. Hanker, I. Master, L. E. Mattison, and B. Witten, *Ibid.* **29**, 82-4 (1957).

Procedure—As a reagent, dissolve 0.2 gram of cobaltous chloride exahydrate in 5 ml. of absolute ethanol and dilute to 100 ml. with anydrous ether. Add 2 drops of 3% hydrogen peroxide solution. To 1 ml. f a solution containing 0.015-0.09 mg. of 1,2-bis(methylthio)ethane in hyl ether add 1 ml. of reagent. Heat at 60-80° for 10 minutes or until ie solvent is evaporated. Cool and add 4 ml. of a Clark and Lubs buffer r pH 1.5 (Vol. I, p. 173) diluted 1:3. Add 0.2 ml. of 0.04% *o*-dianisiine solution in absolute ethanol. Read at 450 mμ after 4 minutes.

THIOPHENE

In the determination of thiophene by isatin in concentrated sulfuric id in the presence of ferric sulfate (Vol. III, p. 502), the color is stable 20°-35° when the reagent has been stabilized by heating.[24] The presice of 0.5% of carbon disulfide does not interfere.

In the determination of thiophene with alloxan (Vol. III, p. 502), the lor gradually changes from violet to red and then to orange as the conntration increases from 0.0025% of sulfur to 0.005%. If the color is ore red or orange than the standard, dilute the sample 20-fold with iophene-free benzene.[25] *p*-Dimethylaminobenzaldehyde in the presence ferric chloride and sulfuric acid is another reagent for thiophene.[26] any impurities in benzene interfere.

Procedure—*By isatin. Benzene.* Add 0.01 gram of isatin and 0.01 im of ferric sulfate to 100 ml. of concentrated sulfuric acid and stabie the reagent by heating at 100° for 15-20 minutes. Cool the reagent. ake 10 ml. of reagent with 1 ml. of a sample such as benzene for 1 nute and read at 530 mμ after 20 minutes.

By alloxan. Benzene. Combine 25 ml. of concentrated sulfuric acid l 25 ml. of 0.05% alloxan solution in concentrated sulfuric acid. Add 1 of sample containing 0.01-0.05% of thiophene, stir for 1 minute, and let stand for 15 minutes. Read at 570 mμ against thiophene-free benzene in the reagent.[27]

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[24] Z. F. Solomko, *Nauch. Zapiski Dnepropetrovsk. Gosudarst. Univ.* **43**, 36-44 53); T. Isobe and O. Akoyoshi, *J. Chem. Soc. Japan* **56**, 817-19 (1953).

[25] K. H. V. French, *J. Appl. Chem.* (*London*) **2**, 664-6 (1952).

[26] J. Kolsek, M. Perpar, and A. Rauschi, *Z. anal. Chem.* **149**, 321-5 (1956).

By p-dimethylaminobenzaldehyde. Benzene. To prepare the reagent, mix 72 ml. of concentrated sulfuric acid, 28 ml. of water, 0.1 gram of *p*-dimethylaminobenzaldehyde, and 0.19 ml. of 5% ferric chloride solution. To a 3-ml. sample, add 4 ml. of reagent and, after 20 minutes, read the lower aqueous phase at 552 mμ against a reagent blank.

MONONITROTHIOPHENE AND DINITROTHIOPHENE

Dinitrothiophene is determined by reaction with ethanol and sodium hydroxide. Mononitrothiophene is quantitatively nitrated to dinitrothiophene, the total dinitrothiophene is determined and the mononitrothiophene is estimated by the difference.[28] A total of the two isomers, 2,4- and 2,5-dinitrothiophene is measured. Dinitrobenzene does not interfere but trinitrobenzene does. The violet color is stable for 10 minutes. The method is applicable for 5 to 500 ppm. of nitrothiophenes.

Procedure—*Nitrobenzene.* To a 5-ml. sample in 5 ml. of 3*A* ethanol, add 0.2 ml. of 1% sodium hydroxide solution and swirl for 15 seconds to develop the color. Read dinitrothiophene at 540 mμ against a mixture of 5 ml. of sample and 5 ml. of 3*A* ethanol.

To 20 ml. of the sample, slowly add with swirling 10 ml. of white fuming nitric acid of approximately 98% concentration. Let stand at room temperature for 15 minutes, cool in ice, and dilute to 50 ml. with water. Draw off the bottom nitrobenzene layer. Wash twice with 25-ml. portions of water and dry over anhydrous sodium sulfate until the layer is free from turbidity. Let the sodium sulfate settle and develop total dinitrothiophene with sodium hydroxide and ethanol following the above procedure. Obtain mononitrothiophene by difference.

p-CHLOROBENZYL *p*-CHLOROPHENYL SULFIDE, CHLORBENSIDE

After *p*-chlorobenzyl *p*-chlorophenyl sulfide is oxidized to the sulfone by chromium trioxide in glacial acetic acid, it forms a stable purple color with *m*-dinitrobenzene with a maximum absorption at 510 mμ.[29] DDT does not interfere.

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[27] Erminio Giovannini, *Ann. chim.* (*Rome*) 43, 736-43 (1953).

[28] W. Leibmann and J. T. Wood, *Anal. Chem.* 29, 1845-6 (1957).

[29] H. J. Hardon, H. Brunink, and E. W. van der Pol, *J. Sci. Food Agr.* 8, 368-70 (1957).

p-Chlorobenzyl *p*-chlorophenyl sulfide is also oxidized with hydrogen peroxide in glacial acetic acid to the sulfone which is then nitrated to the corresponding trinitro derivative. This forms a purple color with sodium methylate in benzene.[30] This compound has absorption peaks at 356 mμ and 575 mμ. The sodium methylate reagent must be standardized to ±0.1% since maximum sensitivity is reached at a concentration of 9.7% and falls rapidly above or below this concentration. Water decreases the sensitivity of the method. Any sulfoxide present is also oxidized to the sulfone.

The results with animal tissues, such as liver, kidney, and muscle, are not very accurate and vary with the quantity of fat present. Ten per cent of DDT in the chlorbenside significantly affects the results. Other fungicides and insecticides including dieldrin, CPBS, parathion, ferbam, aldrin, toxaphene, malathion, γ-BHC, nicotine, and phenyl mercuric salts do not interfere. When present in large quantities, CPCBS and captan give a slight color.

Samples—*Fruit peel.* Peel a sample of approximately 1 kg. and cover he peelings with 250 ml. of hexane. Let stand overnight, decant, and ilter. Distil the solvent, removing the last traces with an air current. Develop the gelatinous residue with *m*-dinitrobenzene.

Leaves. Take one disc of each 50-200 samples with a cork borer. Add 00 ml. of benzene and shake periodically for 30 minutes. Filter and evelop a 1-ml. aliquot with sodium methylate.

Fruit. To a 500-gram sample, add 150 ml. of benzene, cover, and hake for 5 minutes. Filter and develop a 50-ml. aliquot with sodium methylate. In cases of samples with high wax content, during the nitraion step warm the solution gently to allow the initial reaction to subside efore heating at 100°.

Feces. Macerate a 2-gram sample with 100 ml. of benzene, let settle, nd develop 50 ml. of the extract with sodium methylate.

Liver, kidney, and muscle. Macerate a 10-20 gram sample with 100 l. of benzene. Let settle, filter, and develop 50 ml. with sodium methate.

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[30] D. J. Higgins and D. W. Kilbey, *Ibid.* **6**, 441-8 (1955).

Procedure—*By* m-*dinitrobenzene.* To the residue from hexane e traction containing up to 0.4 mg. of *p*-chlorobenzyl *p*-chlorophenyl su fide, add 10 ml. of 5% solution of chromium trioxide in glacial acet acid and heat to boiling for 3 hours. Add small quantities of chromiu trioxide reagent as required to keep the reaction mixture a brownish-re Usually approximately a total of 35 ml. of reagent is required. Cool, d lute with 100 ml. of water, and extract by shaking vigorously for 1 mi ute with 75 ml. of a 1:1 mixture of ether and hexane. Extract the aqu ous layer with a 25-ml. portion of the solvent mixture. Wash the fir extract with 3 portions of water and use these washings to wash t second extract. Filter the combined solvent extracts through anhydro sodium sulfate and distil off the solvent, removing the last traces in gentle current of air. To the residue add 2 ml. of ethanol, 1 ml. of 2 *m*-dinitrobenzene in 95% ethanol, and 1 ml. of 14% potassium hydroxi solution. Swirl gently and incubate at 25° for 1 hour. Cool in a refriger tor for 10 minutes and add 2 ml. of ethanol. Read at 510 mμ.

By sodium methylate. To an aliquot containing 0.05-0.5 mg. of *p*-chl robenzyl *p*-chlorophenyl sulfide, add 2 ml. of 2.5% oleic acid solution benzene to prevent loss of *p*-chlorobenzyl *p*-chlorophenyl sulfide duri oxidation. Concentrate the solution to 5 ml. Add 5 ml. of glacial acet acid and 3 drops of 30% hydrogen peroxide. Let stand for 5 minut and evaporate slowly to 1-2 ml. Add 2 ml. of glacial acetic acid and drops of 30% hydrogen peroxide and evaporate to dryness. Take up a small amount of acetone and evaporate off the acetone at 100°. Wa down any residue on the sides of the tube with acetone and evaporate t acetone finally with an air current. Add 5 ml. of a 1:1 mixture of fumi nitric acid and concentrated sulfuric acid. Heat at 100° for 1 hour nitrate. Cool and add 15 ml. of water to stop the reaction.

Take up in three 15-ml. portions of water followed by three 15- portions of ether. Shake the combined extracts well, separate, and d card the lower layer. Wash the ether layer with 10-ml. portions of 2 sodium hydroxide solution until the washings are alkaline and wa finally with two 10-ml. portions of a saturated sodium chloride soluti Filter the ether layer and wash the filter with three 10-ml. portions ether. Evaporate the ether and remove the last traces with an air curre

Add 5 ml. of dry benzene, cover, and gently swirl to dissolve. To 2-ml. aliquot add 4 ml. of 10% sodium methylate solution in dry me anol. Mix and read at 356 mμ after 1-2 minutes.

3,3′-Dithiobis(2-aminopropionic acid), Cystine

The addition of a divalent sulfur compound to a solution containing
mental iodine and azide catalyzes the reaction resulting in the reduc-
n of iodine to iodide and the evolution of nitrogen. A method for
stine and cysteine is based on their catalytic effect on the reaction
tween iodine and azide. Two moles of cysteine have the same catalytic
ect as one mole of cystine.[31] Cystine bleaches a small amount of iodine
direct reaction independently of its catalytic action and thus causes
calibration curves to depart slightly from linearity. Therefore sam-
s must fall within the concentration range of the standards. Beer's law
followed up to 0.002 M. Because of its poisonous nature the iodine-azide
ution must be handled by an automatic pipet.

A method for cystine is based on the reaction between hydrogen sul-
e, p-phenylenediamine, and ferric chloride to form purple thionine.[32]
e thionine is read in amyl alcohol.

A hydrazinolysis procedure serves as a microdetermination of the
al of cystine and cysteine. The hydrogen sulfide evolved reacts with
methyl-p-phenylenediamine and ferric ammonium sulfate in the pres-
ce of sulfuric acid.[33] Methionine, tyrosine, proline, histidine, and phen-
lanine do not interfere.

Procedure—*By iodine-azide. Proteins.* Hydrolyze a sample contain-
0.002-0.02 mg. of cystine with 0.5 ml. of 2:3 hydrochloric acid by
ating in a sealed glass ampoule at 100° for 10 hours. Evaporate the
drolyzate in the opened ampoule to dryness at 100° using an air jet
speed evaporation. Dissolve the residue in 2 ml. of water and neu-
lize the excess acid by shaking with approximately 5 mg. of dry cal-
m carbonate. Filter to remove humin and excess calcium carbonate.

To prepare the highly poisonous iodine-azide reagent, mix 50 ml. of
% sodium azide solution and 20 ml. of 2.7% potassium triiodide solu-
n. Adjust the pH to 5.8 with 2:3 hydrochloric acid. Dilute to 100 ml.
h water.

Place 5 ml. of the iodine-azide reagent in a water bath at room tem-
ature to prevent excessive temperature fluctuation. Add 0.5 ml. of
nple, mix by lateral shaking, and return to the water bath. Note the
e. Prepare a reagent blank by substituting 0.5 ml. of water for the

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[31] R. D. Strickland, P. A. Mack, and W. A. Childs, *Anal. Chem.* 32, 430-6 (1960).

[32] T. Yoritaka, H. Nakajima, N. Asami, and T. Marsuda, *Nagasaki Igakkai Zasshi*
1376-8 (1953).

[33] K. Kuratomi, K. Ohno, and S. Akabori, *J. Biochem.* (*Tokyo*) **44**, 183-90 (1957).

hydrolyzate. Allow 30-60 minutes for color change depending on sample size as smaller samples require greater incubation time. Since this is a reaction which varies with time, the time and temperature must be rigorously standardized. This may be by running standards at the same time or by use of a calibration curve prepared with the same time of contact of sample and reagent.

Add 0.5 ml. of the solution to a mixture of 5 ml. of water and 5 ml. of carbon tetrachloride. Extract the iodine into the carbon tetrachloride by shaking vigorously for 30 seconds. This stops the reaction. Read the carbon tetrachloride extract at 525 mμ against a water-saturated carbon tetrachloride blank.

By p-*phenylenediamine and ferric chloride.* To a 1-ml. sample containing 0.02-0.1 mg. of cystine, add 1 ml. of 24% sodium hydroxide solution and boil for 5 minutes. Add 1 ml. of 1% *p*-phenylenediamine solution, 1 ml. of 0.1% ferric chloride solution, and 1 ml. of amyl alcohol. Heat to 50-60°, and adjust the pH to about 1 with 1:1 hydrochloric acid. After 30 minutes separate the amyl alcohol and read at 570 mμ. For greater reproducibility extract the solution with amyl alcohol after separation of the first portion and combine the extracts.

*By dimethyl-*p-*phenylenediamine and ferric ammonium sulfate. Cystine and cysteine. Proteins.* Heat the sample containing a total of 10-50 micromoles of *l*-cystine and *l*-cysteine with 0.5 ml. of hydrazine hydrate at 115-120° for 5-7 hours in a hydrazinolyzing apparatus equipped with a condenser and a glass delivery tube. Cool and dilute to 4-5 ml. Add 10 ml. of 1:5 sulfuric acid dropwise and heat at 50-55°. Pass a current of nitrogen at the rate of 2.4-3 liters per hour for 30 minutes. Collect the hydrogen sulfide so distilled in 5 ml. of a reagent containing 6% of zinc acetate, 1.7% of sodium acetate, and 0.005% of sodium chloride. Add 5 ml. of 0.05% dimethyl-*p*-phenylenediamine solution in 1:4 sulfuric acid Add 1 ml. of 12.4% ferric ammonium sulfate solution containing 2.52 ml of concentrated sulfuric acid per 100 ml. of reagent. Let stand for 1 hour dilute 5-10 fold, and read at 630 mμ.

GLUTATHIONE

Glutathione gives a color in the ultraviolet with alloxan.[34] The reaction is inhibited by β-alanine, methionine, and cysteine. Citrate, glycine and other α-amino acids are activators.

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[34] W. W. Kay and K. C. Murfitt, *Biochem. J.* **74**, 203-8 (1960).

Procedure—*Blood.* Dilute 0.2 ml. to 0.8 ml. with water and let stand for 10 minutes to hemolyze. Add 0.8 ml. of 10% trichloroacetic acid solution and centrifuge. To 0.4 ml. add 0.5 ml. of 10% glycine solution and 2.9 ml. of 0.24 M phosphate buffer for pH 7.6. Add 1.2 ml. of a 219:21 mixture of the buffer and 4% sodium hydroxide solution. Add 1 ml. of a solution of alloxan monohydrate containing 1 mg. of the reagent in 1:2000 hydrochloric acid. Read at 305 mμ.

METHIONINE

Methionine is determined by reaction with sodium nitroprusside in alkaline solution, then read in acid solution (Vol. III, p. 498).[35] Sodium salts of arsenate, arsenite, selenate, selenite, and tryptophan do not interfere. If histidine is present add a small volume of glycine solution.

Methionine can be determined in the presence of 3-carboxy-1,3-thiazine by reaction with nitroprusside and extraction of the color due to 3-carboxy-1,3-thiazine with butanol. The aqueous phase containing the methionine is read at 510 mμ.[36] The error is ±5% and the smallest amount which can be measured is 0.6 mg.-%. Tryptophan and to a lesser extent trichloroacetic acid increase the color from 3-carboxy-1,3-thiazine.

Procedure—To a 5-ml. sample containing 0.5-5 mg. of methionine in 1:100 hydrochloric acid add 1 ml. of 57.2% sodium hydroxide solution, 1 ml. of 1% glycine solution, and 0.3 ml. of fresh 10% sodium nitroprusside solution. Incubate at 35-40° for 10 minutes and cool to room temperature. Add 5 ml. of a mixture prepared from 45 ml. of water, 45 grams of concentrated sulfuric acid, and 10 ml. of 85% phosphoric acid. Place in water at room temperature for 10 minutes. Dilute to 15 ml. with water and read at 510 mμ against a reagent blank.

THIOBARBITURIC ACID

The color reaction of thiobarbituric acid with cobalt in strongly alkaline solution is applicable[37] (see 2nd ed., Vol. II, p. 549). The reaction is also applicable to thiourea.

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[35] István Korpáczy, *Magyar Kém. Folyóirat* **60,** 241-3 (1954); E. R. Joson and H. L. Klug, *Proc. S. Dakota Acad. Sci.* **35,** 48-60 (1956).

[36] E. Strack, W. Kunz, and R. Noack, *Z. physiol. Chem.* **313,** 201-11 (1958).

[37] E. Heise and K. H. Kimbel, *Arzneimittel-Forsch.* **5,** 149-51 (1955).

Procedure—Mix 5 ml. of sample in methanol, 4 drops of saturated solution of cobalt chloride in methanol, and a pellet of potassium hydroxide. Mix, let stand for 2 hours, and centrifuge. Read the green solution at 620 mμ.

PHENOTHIAZINES

An analysis of the phenothiazine drugs, promazine, promethazine, and chlorpromazine, is based on the formation of the lauryl sulfate salt of the specific palladium phenothiazine derivative complex.[38] (Cf. Vol. III, p. 505.) The absorption maximum of the palladium-lauryl sulfate complex salts is promazine 545 mμ, chlorpromazine 545 mμ, and promethazine, 525 mμ. This procedure also provides a method of measurement of the oxidative decomposition of the sulfur atom of the compound tested. The following substances in the quantities listed do not interfere with the determination of 5 mg. of promethazine hydrochloride: acetophenetidin 161 mg., acetylsalicylic acid 226 mg., caffeine 32 mg., codeine sulfate 16 mg., meprobamate 200 mg., corn starch 60 mg., lactose 500 mg., magnesium stearate 10 mg., methocel-400 15 mg., and sterotex 2 mg. Phenol and sulfur-containing antioxidants in the concentrations usually present in injection solutions do not interfere. High concentrations of acids, bases, salts, and organic solvents should be avoided.

Samples—*Injection solutions.* Dilute a sample so that 2 ml. contains 0.1-0.125 mg. of phenothiazine derivatives.

Tablets and capsules. Crush the tablets or contents of the capsules and grind into a homogeneous powder. Dissolve an amount of powder equivalent to 10 mg. of the phenothiazine derivative in water and dilute to 200 ml. Filter, discarding the first 150 ml. of filtrate. Develop an aliquot of the remaining filtrate.

Procedure—To prepare the reagent, heat 500 mg. of palladous chloride and 5 ml. of concentrated hydrochloric acid at 100°. Add 200 ml of hot water in small portions with continued heating until solution is complete. Dilute to 500 ml. with water at room temperature. To 25 ml of this solution, add 50 ml. of 8.2% sodium acetate solution and 48 ml of 1:10 hydrochloric acid. Dilute to 500 ml. and mix well as the reagent

To 3 ml. of the buffered palladium chloride solution, add 2 ml. of lution containing 0.1-0.125 mg. of phenothiazine derivative. Mix well ıd add 2 ml. of 0.1% magnesium lauryl sulfate solution. Mix and read ;ainst water at 545 mμ for promazine and chlorpromazine and at 525 μ for promethazine. If the sample is highly colored, use a sample color ank prepared with water and magnesium lauryl sulfate.

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[38] James A. Ryan, *J. Am. Pharm. Assoc.* **48**, 240-3 (1959).

SULFAQUINOXALINE

The total absorbance of sulfaquinoxaline and arsanilic acid is determined by diazotizing and coupling with *N*-1-naphthylethylenediamine. he two components are separated and the arsanilic acid is determined. he difference between this absorbance and total absorbance is that due sulfaquinoxaline.[39]

Procedure—*Feeds.* To a 5-gram ground sample, add 150 ml. of water ıd 5 ml. of 2% sodium hydroxide solution. Heat at 100° for 15 minutes, ol, and dilute to 250 ml. with water. Mix and let settle. To 50 ml. of e supernatant liquid add 3 ml. of concentrated hydrochloric acid and lute to 100 ml. Mix and filter, discarding the first 15 ml. of filtrate if rbid.

To two 10-ml. portions of filtrate add 2 ml. of 0.1% sodium nitrite lution and let stand for 3 minutes. Add 2 ml. of 0.5% ammonium lfamate to destroy excess nitrite and let stand for 2 minutes. Add 1 ml. 0.1% *N*-1-naphthylethylenediamine dichloride solution to the first mple and 1 ml. of water to the second. Mix thoroughly and read after minutes at 545 mμ against water. The sample absorbance—feed abrbance $= x =$ the total absorbance of sulfaquinoxaline and arsanilic id.

Extract 35 ml. of the sample solution with three 50-ml. portions of ıer, shaking rapidly for 20-second intervals each. Aerate the aqueous yer for 10 minutes to remove dissolved ether. Treat two 10-ml. porons of this solution following the above procedure starting with "To o 10-ml. portions. . . ." Then the sample absorbance $-$ feed absorbance $y =$ the absorbance due to arsanilic acid. Then $x - y =$ sulfaquinoxıne.

SULFOBROMOPHTHALEIN

The dye, sulfobromophthalein, is purple in alkaline solution and colors in acid, the difference in intensity of color when the pH is changed from alkaline to acid being proportional to the concentration.[40] This difference is applicable to its determination in serum. p-Toluenesulfonat is added to the buffer to avoid the binding effects of albumin. The pH shift is between 10.4 and 7.1. Over this range there is little or no absorbance change due to turbidity, jaundice, or hemolysis.

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[39] Richard T. Merwin, *J. Assoc. Offic. Agr. Chemists* 39, 307-13 (1956).

Procedure—*Serum.* To prepare the alkaline buffer at pH 10.6-10.7 dissolve 12.2 grams of dibasic sodium phosphate, 1.77 grams of tribasi sodium phosphate, and 3.2 grams of sodium p-toluenesulfonate in 50 ml. of water. Adjust the pH to 10.6-10.7 with 4% sodium hydroxid solution or 1:10 hydrochloric acid. As an acid reagent dissolve 69 gram of monobasic sodium phosphate in water and dilute to 250 ml.

To a 0.5-ml. sample, add 3.5 ml. of the alkaline buffer, mix gently and read at 580 mμ against water. Add 0.1 ml. of the acid reagent, mi gently, and read at 580 mμ against water.

S-(2,5-DICHLOROPHENYLTHIOMETHYL)OO-DIETHYL PHOSPHOROTHIOLOTHIONATE, PHENKAPTONE

The pesticide, *S*-(2,5-dichlorophenylthiomethyl)*OO*-diethyl phosphorothiolothionate, Phenkaptone, is hydrolyzed with sodium hydroxide t form 2,5-dichlorothiophenol. This is brominated in an acid medium an the resulting 2,5-dichlorophenylsulfonylbromide is determined with pyridine and benzidine.[41] The procedure is relatively specific. The sensitivit of the method is 0.005 mg. per 100 grams of sample. Plant waxes are removed with acetonitrile. Carbon disulfide and hydrogen sulfide do no interfere. Other pesticides including dimethyl *S*-(2-ethylthioethyl)phosphorothionate (demeton-*O*), diethyl-p-nitrophenyl phosphorothionat (parathion) and *S*-1,2-di(ethoxycarbonyl) ethyldimethyl phosphorothiolothionate (malathion) interfere slightly.

Procedure—*Fruits and vegetables.* Cover a 500-1000 gram sampl with hexane. Let stand overnight, decant, and filter. Distil off the solven from a 100-ml. portion of the filtrate and remove the last traces of solven in a current of air. Dissolve the residue in hot acetonitrile, cool in a refrigerator, and dilute to 10 ml. with acetonitrile. Filter to remove waxes To a portion of the filtrate containing 0.02-0.2 mg. of parakaptone, ad 35 ml. of 32% sodium hydroxide solution and reflux for 2 hours to hydrolyze the acetonitrile. Steam distil and collect the distillate in 10 ml of methylene chloride in a separatory funnel, keeping the tip of the con

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[40] D. Seligson, J. Marino, and E. Dodson, *Clin. Chem.* 3, 638-45 (1957).

[41] H. J. Hardon, H. Brunink, and E. W. van der Pol, *Analyst* **84**, 102-5 (1959).

enser below the surface of the methylene chloride. Clear the apparatus
f air and carefully add 25 ml. of 3:2 sulfuric acid to the boiling solution.
)istil until 150 ml. of distillate have been collected, or for approximately
0-40 minutes. Shake for 1 minute and allow the layers to separate. Filter
ie methylene chloride layer containing 2,5-dichlorothiophenol through
otton covered with 1 gram of anhydrous sodium sulfate. Collect in a
ask containing 5 drops of 5% mercuric acetate solution in glacial acetic
cid. This prevents evaporation of the 2,5-dichlorothiophenol during the
emoval of the solvent. Repeat the extraction of the distillate and filtra-
on of the extract twice with 10-ml. portions of methylene chloride.
ently evaporate the methylene chloride from the combined extracts at
00°, removing the last traces in a current of air.

As the color reagent, add 10 ml. of concentrated hydrochloric acid to
mixture of 30 ml. of pyridine and 70 ml. of water. Prepare a 5% ben-
idine hydrochloride reagent solution in 1:49 hydrochloric acid. Before
se, mix 3 ml. of the pyridine solution with 0.6 ml. of the benzidine solu-
on.

To the residue, add 0.5 ml. of a 0.8% bromine solution in glacial
cetic acid taking care that the reagent does not touch the neck of the
ask. Let stand for 20 minutes in a refrigerator. Add 0.2 ml. of 8%
henol solution in 5% potassium bromide solution and let stand 2 min-
tes for the phenol to react with the excess bromine. Add 1 drop of 10%
otassium cyanide solution to form cyanogen bromide and after 2 min-
tes, add the pyridine-benzidine reagent. Let stand for 20 minutes in a
efrigerator, dilute to 25 ml. with 95% ethanol, and read after 15 minutes
t 535 mμ.

2,4-DIAMINO-5-PHENYLTHIAZOLE, AMEPHENAZOLE, FENAMIZOL

2,4-Diamino-5-phenylthiazole, precipitated as the reineckate, is read-
y soluble in acetone and gives an absorption curve over the range of
70-580 mμ with a maximum at 525 mμ.[42] The solution is stable for three
ours at room temperature. In a 1.5% 2,4-diamino-5-phenylthiazole so-
ution, 5% of glycerol, 15% of ethanol, 2% of benzyl alcohol, and 60%
f propylene glycol do not interfere. β-Methyl-β-ethylglutarimide does
ot interfere. Polyethylene glycol must be absent. The hydrolysis prod-
cts of 2,4-diamino-5-phenylthiazole do not affect the determination.
eer's law is followed up to 35 mg.

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[42] Per Lundgren. *J. Pharm. and Pharmacol.* **8**, 185-90 (1956).

Procedure—To prepare the reagent, shake 1 part of Reinecke's sal $NH_4[Cr(CNS)_4(NH_3)_2] \cdot H_2O$, with 25 parts of water. Place a 2-15 m sample containing 15-30 mg. of 2,4-diamino-5-phenylthiazole hydrochl ride or hydrobromide in an ice bath and when cooled to the temperatu of the bath, add 5 ml. of reagent. Mix and let stand in the ice bath f 15 minutes. Collect the red precipitate in a sintered glass crucible. Was twice with 2-ml. portions of ice water. Dry the precipitate with suctio Dissolve the precipitate by drawing acetone slowly through the filt under negative pressure and dilute to 25 ml. with acetone. Read at 52 mμ against water.

O,O-DIMETHYL-*S*-(4-OXO-3*H*-1,2,3-BENZOTRIAZINE-3-METHYL)-PHOSPHORO-DITHIONATE, GUTHION

Guthion is hydrolyzed to formaldehyde which is developed wit chromotropic acid.[43] Samples containing oil are extracted with chlorofor and guthion is adsorbed on an alumina column. Washing with pentan elutes the oil after which the guthion is eluted with acetonitrile. Mo of the pigments and other interferences are left on the column. Any re maining oil which passes through the column with the guthion does n interfere.

The guthion in samples containing no oil can be extracted with chlor form. The pigments and other interferences are then removed by filtra tion through decolorizing carbon which has been washed with water an dried, thus eliminating the chromatography step. Any degradation metabolic product of guthion which would yield formaldehyde on hy drolysis and is not removed by chromatography would interfere.

Although 2,4-*D* and related herbicides form formaldehyde on aci hydrolysis, they do not interfere under the conditions of this metho DDT, BHC, chlordan, aldrin, malathion, dieldrin, parathion, demeto schradan, and Phosdrin do not interfere. Thimet, Trithion, Nialat *S,S*-bis(*O,O*-diethyl phosphothionate), and the diethyl homolog guthion liberate formaldehyde upon acid hydrolysis and would interfer

Samples—*Cottonseeds.* To prepare the alumina, mix 800 ml. water and 5 ml. of concentrated hydrochloric acid with 1 pound of 80-20 mesh alumina. Let stand at room temperature for at least 2 hours. Was four times by decantation with water. Filter with suction and continu washing until the filtrate is only very faintly acid. Wash with two 400-m portions of 95% ethanol. Dry at 140° overnight.

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[43] P. A. Giang and M. S. Schechter, *J. Agr. Food Chem.* 6, 845-8 (1958).

Grind whole cottonseeds and extract a weighed portion for 16 hours ith chloroform. Chill the extract and filter with vacuum. Evaporate the olvent at 100° and dissolve the residue in an equal volume of pentane.

Pack 60 grams of activated alumina into a water-cooled 20 × 400 m. chromatographic tube. Prewash by percolation with 100 ml. of entane. When the solvent level reaches the top of the alumina column, dd the oil sample containing up to 0.4 mg. of guthion in 4-5 small ortions. Rinse the flask with nall portions of 125 ml. of pentane and add to the column until l the pentane is used. Rinse the ask with 25 ml. of acetonitrile. hen the pentane reaches the top the column add the acetonitrile. inse and let it run through the lumn. Discard all the pentane llected. Add 100 ml. of acetonitrile to the column and collect all e pentane and acetonitrile that mes through. Evaporate off the lvents carefully at 100° using e flask which originally contained the sample. Evaporate the st trace of acetonitrile in a slow ream of air.

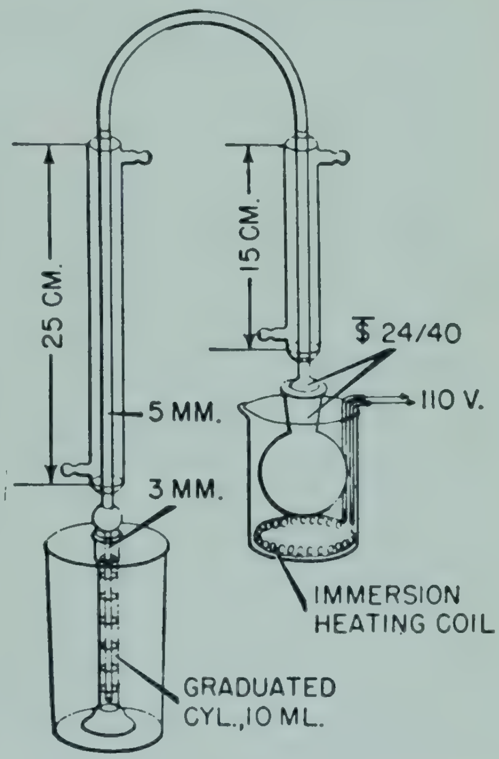

Fig. 21

Steam distillation unit for guthion

For the hydrolysis of guthion, epare a receiver containing 1 ml. water placed in an ice bath. To e residue in the flask add 15 ml. concentrated hydrochloric acid d attach to the distillation unit, gure 21. Reflux in an oil bath r 20 minutes with the water turned on in the 15-cm. condenser. Turn the water and empty the condenser. Turn on the water in the 25-cm. ndenser and distil at 130-160° until the total volume in the receiver 10 ml. Lower the ice bath and receiver so that the tip of the delivery be is just above the surface of the distillate and continue until dis- lation is complete.

Cottonseed oil. Dissolve a 40-gram sample in 40 ml. of pentane and low the procedure for cottonseeds starting at "Pack 60 grams of. . . ."

Procedure—To prepare the chromotropic acid reagent, dissolve 16 grams of the sodium salt of 4,5-dihydroxy-2,7-naphthalenedisulfonic acid, chromotropic acid, in concentrated sulfuric acid with heating. Cool to room temperature and dilute to 1 liter. Store in a dark bottle.

Cool 2 ml. of the distillate in a glass-stoppered test tube in an ice bath. Slowly add 3 ml. of the chromotropic acid reagent under a hood. Mix by gentle swirling and tapping. Stopper loosely with a glass stopper and heat for 1 hour at 100°. Cool rapidly in water and dilute to 5 ml. with concentrated hydrochloric acid. Mix and read at 570 mμ against a reagent blank for which the chromotropic acid reagent has been heated at 100° for 1 hour.

5-*n*-HEPTYL-2-THIOHYDANTOIN

A fluorometric method for 5-*n*-heptyl-2-thiohydantoin uses 2,6-dichloroquinonechloroimide as the reagent.[44] The maximum color development occurs within a narrow pH range.

The choice of a buffer is critical. In the presence of a borate buffer at pH 8.7, a purple-blue color forms between 5-*n*-heptyl-2-thiohydantoin and 2,6-dichloroquinonechloroimide with no fluorescence, while a brownish-violet color forms in the presence of an ammonia-ammonium chloride buffer at pH 8.7 with a strong green fluorescence under ultraviolet light An isopropanol-isobutanol mixture is used to keep the fats in solution Hydantoins containing oxygen instead of sulfur in the 2-position develop no measurable fluorescence. Thiouracil and 6-propyl thiouracil do not develop fluorescence.

Procedure—*Water, urine, spinal fluid, and plasma.* To prepare a buffer for pH 6 dissolve 675 grams of monobasic potassium phosphate in 3 liters of water. Dissolve 150 grams of dibasic sodium phosphate, in 500 ml. of water. Mix 11 parts of the potassium phosphate solution with 14 parts of the sodium phosphate solution, varying the ratio if necessary to adjust to pH 6.

To 8 ml. of chloroform in a separatory funnel which has the stopcock lubricated only with water, add a sample containing up to 0.048 mg of 5-*n*-heptyl-2-thiohydantoin. Add 2 ml. of the pH-6 buffer and dilute to 5 ml. with water. Shake for 2 minutes, allow the layers to separate, and draw off the chloroform layer. Centrifuge to clarify.

To prepare the dichloroquinonechloroimide reagent, dissolve 1 gram in 50 ml. of acetone. While stirring rapidly, add water slowly until permanent precipitate forms. Filter with suction, dry briefly with suction, and then dry in a vacuum chamber. Dissolve 50 mg. in 250 ml. of isopropanol. To prepare the pH-10.2 buffer, dissolve 23 grams of ammonium chloride in 100 ml. of water and adjust the pH to 10.2 with concentrated ammonium hydroxide. Evaporate the chloroform from a 5-ml. portion of the sample solution in the fluorometer tube and cool. Add 4 ml. of a 3:1 mixture of isopropanol and isobutanol. Add 2 ml. of water, then 0.5 ml. of pH-10.2 buffer and 0.5 ml. of dichloroquinonechloroimide reagent. Mix, let stand for 2 hours, and read in a fluorometer which has been set with a quinine reference standard containing 0.1 mg. of quinine sulfate per liter of 1:350 sulfuric acid.

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[44] M. E. Auerbach and E. Angell, *J. Pharm. and Pharmacol.* 10, 776-9 (1958).

If fat is known to be absent the development of fluorescence is simplified. Take up the residue from chloroform in 1 ml. of 95% ethanol and add 5 ml. of water. Add 0.5 ml. of pH-10.2 buffer and 0.5 ml. of reagent. Fluorescence is fully developed in an hour. The intensity is slightly greater in the absence of isopropanol and isobutanol.

TETRAMETHYLTHIURAM DISULFIDE, BIS(DIMETHYLTHIOCARBAMYL DISULFIDE)

Tetramethylthiuram disulfide, an active ingredient in fungicides and an accelerator in rubber vulcanization, is developed as the chloroform-soluble brown copper salt with cuprous chloride[45] (Cf. Vol. III, p. 478). This reaction is similar to the determination of diethyldithiocarbamic acid (Vol. III, p. 476). Although dithiocarbamates such as ferbam, ziram, and nabam develop a brown color with cuprous chloride in chloroform, the curve of tetramethylthiuram disulfide is distinguishable from these dithiocarbamates.

Tetramethylthiuram disulfide reacts rapidly in aqueous solution with copper sulfate and sodium thiosulfate. The resulting color is brownish-purple to purple.[46] It is unstable to sunlight and heat. The color is neither stabilized by gelatin nor does it pass through a cellophane membrane. A method for reading in ultraviolet is given on page 471.

Sample—*Whole corn and ground-feed mixes.* To a 200-gram corn sample or a 100-gram feed mixture or corn-meal sample, add 100 ml. of chloroform. Use 200 ml. of chloroform for samples of finely divided feeds or ground corn. Stopper and shake intermittently for 5 minutes. Filter and treat a 50 ml. portion of the extract as follows.

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[45] George E. Keppel, *J. Assoc. Offic. Agr. Chemists* **39,** 708-12 (1956).

[46] T. Iijima and T. Eguchi, *Nippon Gomu Kyokaishi* **29,** 551-5 (1956).

1. For extracts with no color or a small amount of color as in most whole corn extracts add 5 grams of anhydrous sodium sulfate. Shake for 5 minutes and filter.
2. For extracts of moderate color as in low-grade corn samples containing much broken or damaged corn, add 5 grams of a 3:1 mixture of Attapulgus clay and anhydrous sodium sulfate. Shake intermittently for 5 minutes and filter.
3. For extracts with a large amount of color such as in ground mixes, especially those containing alfalfa, add 50 ml. of petroleum ether and 5 grams of a 3:1 mixture of Attapulgus clay and sodium sulfate. Shake intermittently for 5 minutes, filter, and evaporate 50 ml. of the filtrate at 100° just to dryness. Take up the residue in chloroform and dilute to 25 ml. with chloroform.

Procedure—To a 15-ml. portion of a chloroform extract of the sample containing 0.004-0.032 mg. of tetramethylthiuram disulfide per ml., add 10 mg. of cuprous chloride and mix by swirling for 1 minute. Filter and read at 420 mμ against the extract of the sample as the blank.

In the ultraviolet. See rubber accelerators.

BIS(DIETHYLTHIOCARBAMYL)DISULFIDE, DISULFIRAM

Disulfiram, a rubber accelerator and seed disinfectant, develops a yellow color with a maximum absorption at 434 mμ upon reaction with copper salts in polar or nonpolar solvents.[47] The color is specific for the group :NCSSSCSN:. Tetraethylthiuram monosulfide and bis(ethyl) xanthogen do not develop color with the reagent. Beer's law is followed for 0.25-0.75 mg.

Procedure—*Aqueous solutions.* To a 10-ml. sample containing 0.2-1 mg. of disulfiram per 100 ml. add 0.45 gram of copper turnings which have been treated with nitric acid and washed with ethanol and ether. After 7 hours read at 430 mμ.

Methanol solutions. Follow the procedure for aqueous solutions, using 0.2 gram of copper turnings and read after 5-6 hours.

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[47] B. Salvesen and L. Domange, *Ann. pharm. franc.* 13, 499-502 (1955).

RUBBER ACCELERATORS

The rubber accelerators mercaptobenzothiazole, benzothiazyldisulfide, 2-mercaptothiazoline, tetramethylthiuram disulfide, tetramethylthiuram monosulfide, diphenylguanidine, and di-*o*-tolyguanidine are read in choroform.[48] Beer's law is followed over an optical density range of 0.3-0.6. In the light tetramethylthiuram monosulfide is partially oxidized to tetramethyl disulfide and benzothiazyl disulfide is reduced to mercaptobenzothiazole. Therefore, when these accelerators are analyzed, the solutions should be kept in the dark.

Samples containing a mixture of 2 accelerators are analyzed by reading in chloroform at two wave lengths and calculating percentages by simultaneous equations based on the absorption maximum of each component, or by analysis of the ratio of absorption at the wave length of one component and at the isosbestic point, the point where the curve of each component crosses. Natural rubber, smoked sheet, shows a weak absorption at 270-290 mμ and GR-S shows maxima at 262 mμ and 290 mμ. However, their interference is negligible.

A method of development of tetramethylthiuram disulfide is given on page 470.

Procedure—Weigh a 4-8 mg. master batch sample and shake for 20 minutes with chloroform to dissolve. Dilute to 50-100 ml. with chloroform and read at the following wave lengths:

mercaptobenzothiazole	329 mμ
benzothiazyl disulfide	274 mμ
2-mercaptothiazoline	280 mμ
tetramethylthiuram disulfide	282 mμ
tetramethylthiuram monosulfide	282 mμ
diphenylguanidine	258 mμ
di-*o*-tolylguanidine	252 mμ.

Read mixtures of benzothiazyl disulfide and diphenylguanidine at 258 mμ and 274 mμ, mixtures of mercaptobenzothiazole and di-*o*-tolyguanidine at 274 mμ and 329 mμ, mixtures of 2-mercaptothiazoline and tetramethylthiuram monosulfide at 280 mμ and 291.5 mμ.

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[48] K. E. Kress, *Anal. Chem.* 23, 313-22 (1951).

4-ACETAMIDOBENZALDEHYDE THIOSEMICARBAZONE

Naphthylenediamine is a reagent for 4-acetamidobenzaldehyde thiosemicarbazone. The color is stable for 2 hours and the sensitivity of the method is to 0.1 mg. per 100 ml.[49]

Procedure—*Serum.* Dilute a 1-2 ml. sample to 5 ml. Add 5 ml. of 20% trichloroacetic acid solution, shake, and heat at 90-95° for 30 minutes. Cool to 20-30°, restore the evaporated water, and filter. To 5 ml. of the filtrate, add 1 ml. of 0.1% sodium nitrite solution. Shake and let stand for 20 minutes. Add 1 ml. of 1% ammonium sulfamate solution. Shake and add 1 ml. of 0.1% naphthylethylenediamine solution. Let stand for 15 minutes and filter if necessary. Read at 550 mμ.

Urine. Dilute 1:5 to 1:10 and follow the procedure for serum.

p-HYDROXYBENZYL THIOCYANATE

p-Hydroxybenzyl thiocyanate, sinalbin mustard oil, is determined as ferric thiocyanate.[50]

Procedure—*Fodder and forage plants. Seeds.* To hydrolyze *p*-hydroxybenzyl thiocyanate, keep 25 mg. of crushed fat-free seeds moist for 2 hours. Dilute to 20 ml. with methanol, and shake occasionally for 2 hours. Centrifuge for 5 minutes.

To a 15-ml. aliquot in dim light, add 0.3 ml. of 1% sodium hydroxide solution in methanol and shake. Add 0.6 ml. of 1% ferric chloride solution in methanol and shake. Add 0.5 ml. of 1:59 hydrochloric acid in methanol and dilute to 25 ml. with absolute methanol. Read at 550 mμ. Calculate *p*-hydroxybenzylthiocyanate by multiplying the extinction by 1.72.

Fresh stems and leaves. Extract 0.5-2 gram samples for 10 hours with ether. Moisten for 2 hours and mix with 2.5-10 grams of anhydrous sodium sulfate. Extract with ether for 4 hours and evaporate adhering ether. Wash the residue with methanol and develop the extract as for seeds from "To a 15-ml. aliquot. . . ."

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[49] M. Filomeni, R. Giuliano, and F. S. Trucco, *Atti. soc. lambarda sci. med. biol.* **5**, 196-7 (1949-50).

[50] K. Scharrer, J. Jung, and A. Pilgenrother, *Arch. Tierernahr., Beihefte* **No. 5**, 77-94 (1954).

Very young plants. Treat with 0.3 ml. of 1% sodium hydroxide solution in methanol and 1 ml. of a saturated barium hydroxide solution in methanol. Dilute to 20 ml. with methanol and filter. Make a portion of the filtrate colorless to phenolphthalein with 1:59 hydrochloric acid in methanol. Add 0.6 ml. of 1% ferric chloride solution in methanol, dilute to 25 ml. with methanol, and read at 550 mμ.

Toluene Diisocyanates

Both toluene-2,4-diisocyanate and toluene-2,6-diisocyanate are determined by hydrolysis to the corresponding diamine, diazotization, and coupling with *N*-1-naphthylethylenediamine.[51] The absorption maximum of solutions containing both isomers is at 550 mμ, the absorption maximum of toluene-2,4-diisocyanate is at 550 mμ, and that of toluene-2,6-diisocyanate is at 560 mμ. Phenol and the urea derivative, 3,3′-diisocyanate-4,4′-dimethylcarbanilide do not interfere. Aromatic amines interfere. Beer's law is followed up to 0.16 mg., and as little as 0.01 ppm. of toluenediisocyanate can be measured.

Procedure—*Air.* As the absorber medium, add 35 ml. of concentrated hydrochloric acid and 22 ml. of glacial acetic acid to 600 ml. of water and dilute to 1 liter. Assemble an all-glass bubbler with 1-mm. impinger opening connected to a pump. Add 15 ml. of absorber medium and collect the sample at the rate of 0.1 cubic foot of air per 3 minutes. Add 0.5 ml. of a 3% sodium nitrite solution containing 5% of sodium bromide as a catalyst. Agitate gently and let the solution stand for 90 minutes. Add 1 ml. of 10% sulfamic acid to destroy excess nitrous acid and let stand for 2 minutes. Add 1 ml. of 0.1% *N*-1-naphthylethylenediamine hydrochloride solution in 1:50 hydrochloric acid. Agitate and after 5 minutes for color development, dilute to 20 ml. with water. Read at 550 mμ against a reagent blank.

3,3′-Diisocyanate-4,4′-dimethylcarbanilide

Both 3,3′-diisocyanate-4,4′-dimethylcarbanilide and toluenediisocyanates form a yellow color with ethyl Cellosolve and sodium nitrite.[51] Boric acid enhances the color formed with the carbanilide. In order to determine the carbanilide in the presence of toluenediisocyanate, the latter must be measured first by the diazometric method and the carbanilide measured by difference. Acetic acid, *o*-dichlorobenzene, dimethylformamide, and xylene must be absent. Aromatic amines may interfere. Beer's law is followed up to 0.16 mg.

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[51] Kalman Marcali, *Anal. Chem.* **29**, 552-8 (1957).

Procedure—*Air.* As an absorber medium, dissolve 5 grams of sodium nitrite and 10 grams of powdered boric acid in 200 ml. of water. Dilute to 1 liter with ethyl Cellosolve. Transfer 25 ml. of the absorber medium into an impinger flask, assemble the impinger bubbler, and connect to a pump. Collect a 0.3-0.8 cubic foot sample at the rate of 0.1 cubic foot of air per 3 minutes. Disconnect the pump and let stand for 1 hour. Dilute to 25 ml. with absorber medium and read at 450 mμ against the absorber medium, to give a total of toluene diisocyanate and carbanilide present. Determine toluene diisocyanate by the diazometric procedure and calculate 3,3′-diisocyanate-4,4′-dimethylcarbanilide as follows:

$$\left(A - \frac{VB}{N}\right)\frac{0.79}{MV} = \text{ppm. of 3,3}'\text{-diisocyanate-4,4}'\text{-dimethylcarbanilide}$$

where

A = micrograms of total toluene diisocyanate and carbanilide found by the Cellosolve method

V = sample size in cubic feet used in the Cellosolve method

N = sample size in cubic feet in the diazometric method

B = micrograms of toluene diisocyanate found by the diazometric method

M = 322, the molecular weight of 3,3′-diisocyanate-4,4′-dimethylcarbanilide.

PROMETHAZINE, PHENERGAN

The color developed by promethazine with sulfuric acid in about 70% solution is suitable for reading.[52]

Procedure—*Blood.* Oxalate a 5-ml. sample with 0.3 ml. of 2% potassium oxalate solution. Add 4 ml. of concentrated hydrochloric acid and 1 ml. of water. Heat for 4 hours at 100°, cool, and dilute with 20 ml. of water. Adjust the pH to 6-7 with sodium carbonate and add 2 ml. of 20% sodium carbonate solution. Extract 3 times with 20-ml. portions of peroxide-free ether and wash the combined ether extracts with 0.7% sodium carbonate solution and then with water. Shake with 5 ml. of

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[52] Juan Lavarello Di Tolla, *Anales fac. farm. y. bioquim., Univ. nacl. mayor San Marcos (Lima)* **7**, 360-7 (1956).

1:350 sulfuric acid. Heat the aqueous phase a few minutes at 60° to expel the ether. Treat a 2.5-ml. portion of the solution with 7.5 ml. of concentrated sulfuric acid and read at 540 mμ.

2-THIOURACIL

2-Thiouracil is developed with sodium nitroprusside and hydroxylamine hydrochloride.[53] The method is applicable to urine or deproteinized serum. The same technic is applicable to 4-methyl-2-thiouracil and to 4-propyl-2-thiouracil.

Procedure—To prepare the reagent, add 5 ml. of 10% hydroxylamine hydrochloride solution to 10 ml. of 5% sodium nitroprusside solution. Shake and after 2 minutes add 10 ml. of 10% sodium bicarbonate solution. Shake and after 10 minutes add 0.11 ml. of bromine. Shake and after 10 minutes add 5 ml. of 2% phenol solution. Age at room temperature for 24 hours during which time the reagent turns green. Filter before using.

Prepare a buffer at pH 6.8 by mixing equal parts of 2.8% dibasic sodium phosphate solution and 2.7% monobasic potassium phosphate solution. To 19 ml. of buffer, add 1 ml. of reagent. Heat to 50°, and add 4 ml. to 4 ml. of water as a blank. Add 4 ml. to a 4-ml. sample at 50°. Maintain both the blank and sample at 50° for 5 minutes and read at 660 mμ.

6-PROPYL-2-THIOURACIL

Ruthenium chloride reacts with the thiocarbamide linkage in 6-propyl-2-thiouracil to produce a product read at 520 mμ.[54]

Procedure—Treat a 0.2-0.9 ml. sample containing 0.02-0.08 mg. of 6-propyl-2-thiouracil per ml. in concentrated hydrochloric acid with 1.5 ml. of 0.1% ruthenium chloride solution. Heat for 90 minutes at 100°, dilute to 10 ml. with 1:2 hydrochloric acid, and read at 520 mμ.

DIAMINODIPHENYLSULFONE

From 0.01 to 1 mg. of diaminodiphenylsulfone per 100 ml. of sample is developed with 1-sulfomethylaminonaphthalene-8-sulfonic acid.[55]

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[53] W. Doden and R. Kopf, *Arch. exptl. Path. Pharmakol.* **213**, 51-8 (1951).

[54] F. Reinhardt, *Hoppe-Seyler's Z. physiol. Chem.* **293**, 268-72 (1953).

[55] M. Schoog, *Arzneimittel Forsch.* **2**, 512-4 (1952).

Procedure—*Blood.* Hemolyze a 1-ml. sample with 9 ml. of water. Heat and add 5 ml. of 10% trichloroacetic acid solution. Filter. To a 5-ml. aliquot containing 0.01-1 mg. of diaminodiphenylsulfone per 100 ml., add 1 ml. of 0.1% sodium nitrite solution. After 15-20 minutes add 1 ml. of 0.5% sulfamic acid. Mix with 1 ml. of 0.1% 1-sulfomethylaminonaphthalene-8-sulfonic acid and read the red color at 530 mμ.

PHENOLSULFONIC ACID

Phenolsulfonic acid is read in the ultraviolet in a slightly alkaline medium after extraction of the sample with chloroform to remove interferences.[56] Beer's law is followed for 5-300 mg. per liter.

Procedure—*Antiperspirants containing no sulfated surface-active agents.* Take up a sample containing 5-10 mg. of phenolsulfonic acid with 30 ml. of water and acidify with concentrated hydrochloric acid. Extract with three 30-ml. portions of chloroform. Discard the chloroform extracts and filter the aqueous solution. Dilute the filtrate to 100 ml. with water. Neutralize a 10-ml. aliquot to litmus paper with 10% sodium hydroxide solution and add 4 ml. in excess. Dilute to 100 ml. with water and read at 253 mμ against 0.4% sodium hydroxide solution.

Antiperspirants containing a sulfated surface-active agent. Reflux a sample containing 5-10 mg. of phenolsulfonic acid with 10 ml. of water and 2 ml. of concentrated hydrochloric acid for 30 minutes. Cool to room temperature and mix with 20 ml. of water. Follow the procedure for antiperspirants containing no sulfated surface-active agent starting at "Extract with three 30-ml. portions of chloroform."

DODECYLBENZENE SODIUM SULFONATE

Dodecylbenzene sodium sulfonate is developed with methylene blue. In canned foods 25 ppm. or 0.0025% can be detected and larger amounts estimated.[57] Extraction may be with alcohol or aqueous alkali. Peas, which form a green color with methylene blue, are precipitated by making the juice alkaline and heating, or are extracted with hexane. Peach pulp, which also causes a green color with methylene blue, is acidified and evaporated to remove the interfering material. Chlorophyll is extracted with hexane.

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[56] John E. Clements, *J. Assoc. Offic. Agr. Chemists* 37, 798-801 (1954).

[57] J. C. Harris and F. R. Short, *Food Technology* 6, 275-8 (1952).

Sample—*Canned foods.* Separate the juice and pulp of canned food. Filter the juice. If the total surface-active agent is desired, weigh the pulp and obtain the density of the liquid portion by weighing a measured volume. To 20 ml. of the juice add 80 ml. of absolute ethanol, cover, and boil about 45 minutes. Cool, filter, and rinse with 80% ethanol until the rinsings are colorless. Cover and evaporate the filtrate to 50 ml. Rinse the cover and sides of the beaker with hot water and dilute to 100 ml. Add 0.5 ml. of hydrochloric acid and evaporate to about 35 ml. Cool, filter, rinse with several small portions of water, and dilute to 50 ml.

Cut the pulp into small pieces and mash thoroughly. To 20 grams of pulp add 300 ml. of 80% ethanol. Cover and boil for 45 minutes. Cool, filter, and rinse thoroughly with 80% ethanol. Cover and concentrate the filtrate to 75 ml. Rinse the cover and sides of the beaker with hot water, dilute to 150 ml. and add 1 ml. of concentrated hydrochloric acid. Evaporate to 75 ml. and cool. Filter and dilute to 100 ml. with water. Develop the samples from the juice and the pulp separately.

As an alternative the juice is prepared by alkaline treatment. Add 1 ml. of 28% sodium hydroxide solution to 20 ml. of juice and heat at 100° for 1.5 hours to precipitate interfering materials. Do not allow the volume of liquid to fall below 15 ml. Cool and filter. Neutralize with hydrochloric acid and dilute to a known volume.

As an alternative method cut the pulp into small pieces and extract with 100 ml. of 95% ethanol containing 1 ml. of concentrated hydrochloric acid until the color is nearly gone from the pulp. This usually requires 1.5-2 hours. Concentrate the extract to 30 ml. and add 30 ml. of water. Neutralize with sodium hydroxide solution and filter. Rinse several times and dilute to 100 ml. Remove chlorophyll by four 20-ml. extractions with hexane. Wash the hexane with water and add the washings to the sample solution. Evaporate the solution to 75 ml. and filter. Wash and dilute the filtrate to 100 ml.

Procedure—Dilute a sample containing up to 0.4 mg. of dodecylbenzene sodium sulfonate to 24 ml. Acidify with 4-6 drops of concentrated hydrochloric acid and gently shake for 1 minute with 1 ml. of 0.1% methylene blue chloride solution. Extract the methylene blue complex 3 times with 25-ml. portions of chloroform. Draw each extract into a second separatory funnel and wash with 1:1000 hydrochloric acid. Filter the chloroform extracts through cotton and dilute the combined extracts to 100 ml. with chloroform. Read at 652 mμ against a reagent blank.

XANTHATE SULFUR

The concentration of xanthate in viscose is expressed as the per cent of xanthate sulfur or as the γ value, which is the degree of substitution of the cellulose multiplied by a factor of 100.[58] The viscose is forced through a basic ion-exchange column to remove interference from other sulfur-containing ions, including the trithiocarbonate ion, perthiocarbonate ion, and sulfide ion. Reading is then in the ultraviolet. The molar extinction coefficient of cellulose xanthate must be known to calculate the percentage of xanthate sulfur. This is obtained by calibrating the procedure using viscose of known cellulose xanthate concentration as determined by the gravimetric sulfate method. The xanthate sulfur is then determined by difference from a determination of total sulfur and the by-product sulfur in the viscose. The average value of 19 such determinations gives an extinction coefficient of 15,940. The value of 15,900 is used for calculations.

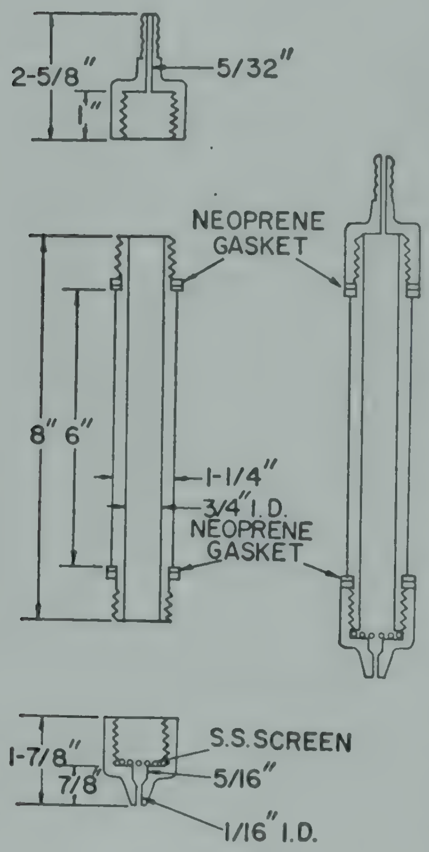

FIG. 22

Stainless steel high-pressure ion-exchange column for preparation of viscose samples

Samples containing large quantities of sulfur by-products which are removed by the ion-exchange resin present larger errors than other samples as the calculations as based on the viscose weight after passage through the column. However, the error from viscose samples containing 2% carbon disulfide, the extreme limit in commercial viscoses, is less than 1.5%. The reading should be taken rapidly as the viscose decomposes rapidly in alkaline media.

Alkyl xanthates are read in alkaline solution at 301 mμ.[59] For this adjust the pH to 9.9 with a buffer containing 0.21% of sodium bicarbonate and 0.265% of sodium carbonate. A pH above 10 or below 9 accelerates decomposition. *n*-Alkyls from methyl through amyl absorb at the same wave length. Nephelometric estimation of butyl xanthate in the presence of excess nickel ion has also been suggested.[60]

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[58] J. P. Dux and L. H. Phifer, *Anal. Chem.* 29, 1842-5 (1957).

[59] P. J. C. Fierens, J. Adam, and E. Royers, *Compt. rend. congr. intern. chim. ind. 31e, Liège 1958, Ind. Chim. belge, Suppl.* 2, 777-80.

Procedure—Assemble the apparatus shown in Figure 22. Place a 60-mesh piece of stainless steel screening in the bottom to prevent forcing of the resin beads through the column. Prepare the Amerlite IRA 400 or any strong-base resin, 20-50 mesh, either in the chloride or hydroxide form, by drying with suction. Add 6 grams of resin to the column and pack tightly. Add 6-10 ml. of viscose and screw on the top of the apparatus. Apply air pressure of 30-40 pounds to force the sample through the resin and discard the first 3-4 ml. of viscose. Collect 1-3 drops of viscose in a weighed flask containing 20 ml. of 0.4% sodium hydroxide solution. Weigh to determine the weight of viscose and shake thoroughly to dissolve. Dilute to 50 ml. with 0.4% sodium hydroxide solution and dilute a 5-ml. aliquot to 250 ml. with 0.4% sodium hydroxide solution. If the reading is not made immediately store the solution in an ice bath before the final dilution. Read at 303 mμ against sodium hydroxide.

Calculate % xanthate sulfur and γ value as follows:

$$\%\ \text{xanthate sulfur} = \frac{1.005 \times \text{absorbance at 303 m}\mu}{\text{sample weight}}$$

$$\gamma\ \text{value} = \frac{2.545 \times \text{absorbance at 303 m}\mu}{\text{sample weight} \times \%\ \text{cellulose in viscose}}.$$

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[60] I. N. Plaksin, N. A. Suvorovskaya, and M. M. Voskresenskaya, *Sbornik Nauch. Trudov. Moskov. Inst. Tsvetnykh Metal. i Zolota* **1958,** No. 31, 305-7.

CHAPTER 19

HALOGEN COMPOUNDS

DETERMINATION of trichloroacetic acid appears under substituted monobasic aliphatic acids, page 320. The determination of trichloroethanol is described in a procedure for trichloro compounds, page 483. Hydrolysis of urocholalic acid yields trichloroethanol but not in quantitative yields.[1] Fluoroorganic compounds in air are burned in a quartz tube at 900° to silicon tetrafluoride. The latter is absorbed in water and the silicon determined as molybdenum blue (Vol. IIIA, pp. 581-6).[2]

Naphthalene, 1-chloronaphthalene, and 2-chloronaphthalene are determined in the presence of each other by reading at 310, 314, and 320 mμ and solving simultaneous equations.[3] Highly chlorinated naphthalenes in petroleum products are determined by the color with dimethylaniline (Vol. III, page 523) read in hexane solution at 400 mμ.[4] Passage through a column of activated alumina and Supercel removes interfering pigments. Chlorothiazide, diuril, is read over the range of 200-350 mμ.[5]

2-Amino-5-chlorobenzoxazole, zoxazolamine, is read fluorimetrically at 320 mμ and pH 11 when activated at 280 mμ.[6] Similarly oxychloroquine is read at 380 mμ and pH 11 when activated at 335 mμ. 3-Chloro-7-methoxy-9-(1-methyl-4-diethylaminobutyl-amino)acridine, quinacrine, is activated at 285 and 420 mμ and pH 11 for reading at 500 mμ.

Halogen-substituted phenols are determined by titanium sulfate, page 95. Their wave lengths of maximum absorption are *p*-bromophenol 473 mμ, *p*-chlorophenol 465 mμ, and 2,4-dichlorophenol 490 mμ. For determination of chlorobenzene as 2,4-dinitrophenol in the presence of benzene see page 12. For trichlorophenol see page 99.

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[1] J. Kubis and N. Lišková, *Bratislav. Lekárské Listy* **36,** Pt. 1, 153-60 (1956).

[2] E. A. Peregud and B. S. Boĭkina, *Zhur. Anal. Khim.* **8,** 178-81 (1953).

[3] A. P. Kreshkov, Yu. Ya. Mikhaĭlenko, and I. P. Smirenkina, *Ibid.* **13,** 242-5 (1958).

[4] R. W. Engel, N. O. Price, W. N. Linkous, and W. B. Bell, *Virginia Agr. Expt. Sta., Virginia Polytech. Inst. Tech. Bull.* **121,** 7 pp. (1953).

[5] G. Ferrari and E. Marcon, *Boll. chim. farm.* **97,** 385-8 (1958).

[6] S. Udenfriend, D. E. Duggan, B. M. Vasta, and B. B. Brodie, *J. Pharmacol. Exptl. Therap.* **120,** 26-32 (1957).

PHOSGENE, CARBONYL CHLORIDE

The reaction of phosgene with aniline in aqueous solution produces 1,3-diphenylurea, carbanilide (Vol. III, p. 511). Small quantities of 1,3-diphenylurea can be determined in the presence of large amounts of aniline in acidic methanol. Both compounds exhibit an absorption maximum at 254.5 mμ but the absorbance of the 1,3-diphenylurea on a weight basis is 93.6 times as intense as that of aniline.[7] The sharp absorption peak of aniline at 260.5 mμ is appropriate for determination of the amount of unreacted aniline in order to calculate a correction.

When an ether solution of 4-(4-nitrobenzyl)pyridine is added to an ether solution of phosgene, the compound $[4\text{-}(4\text{-}O_2NC_6H_4CH_2)C_5H_4NCl]_2CO$ forms which is soluble in water and gives a violet color with alkali. Phosgene in concentrations up to 0.005 mg. per ml. in the atmosphere can be determined by passing the air through acetone oxime or diethyl phthalate containing 4-(4-nitrobenzyl)pyridine and reading at 415 mμ.[8]

Procedure—*By aniline.* Prepare an aniline solution so that 50 ml. contains 2 mg. for each mg. of phosgene anticipated, plus an excess of 50 mg. Bubble a gas sample through the aniline reagent at a rate such that not more than 2 mg. of phosgene is passed in per minute. Take up the solution and any precipitate with methanol. Add 2 ml. of concentrated hydrochloric acid. Dilute with methanol to 250 ml. and dilute an appropriate aliquot to 100 ml. with methanol. Determine the net absorbance at 260.5 mμ and multiply by the absorbance ratio R. This value of R is about 2.8, the absorbance of aniline at 254.5 mμ as compared with that at 260.5 mμ. Subtract the result from that at 254.5 mμ to obtain the absorbance due to 1,3-diphenylurea. Multiply this by 0.615 mg. of 1,3-diphenylurea per 100 ml. per unit of absorbance in a 1-cm. cell. Multiply by 0.466 to convert 1,3-diphenylurea to phosgene.

CHLOROFORM

The reaction of chloroform with pyridine in the presence of strong alkali and heat (Vol. III, pp. 511-3) is applicable to its determination as traces in carbon tetrachloride.[9] With various modifications the reaction is applicable to carbon tetrachloride, chloral hydrate, trichloroethylene, trichloroacetic acid, and trichloroethanol. The pyridine-water ratio is a critical factor, as the concentration of pyridine directly influences the intensity of color produced. Interfering substances under the conditions specified here are 1,1,1,2- and 1,1,2,2-tetrachloroethane, pentachloroethane, and 1,1-dichloroethylene. Methylene chloride ethyl chloride, 1,1-dichloroethane, 1,2-dichloroethane, 1,1,1-trichloroethane, 1,1,2-trichloroethane, hexachloroethane, and perchloroethylene can be tolerated. The presence of 20 ppm. of carbon disulfide interferes and may be detected by the instant yellowing of the mixture before it is placed in the boiling water bath. The color developed decreases with time.

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[7] Warren B. Crummett, *Anal. Chem.* **28,** 410-2 (1956).

[8] Andre Lamouroux, *Mem. poudres* **38,** 383-6 (1956).

[9] Charles D. Hildebrecht, *Anal. Chem.* **29,** 1037-9 (1957).

Sample—*Carbon tetrachloride.* To a 1-ml. sample containing 10-900 ppm. of chloroform, add 5 ml. of water and 5 drops of 10% sodium hydroxide solution. Add 15 ml. of pyridine, mix well, and heat in water at 100° for 3 minutes ±5 seconds. Cool at 15-20° for 7 minutes and dilute to 100 ml. with methanol. Mix well. Read after 10 minutes at 525 mμ against water.

TRICHLOROETHYLENE

The reaction of pyridine in hot alkaline solution to produce a red color with halogenated compounds (Vol. III, pp. 514-5) is applied to trichloroethylene.[10] The absorbance is decreased by substitution of xylene as a solvent, by addition of more or less than 0.35 ml. of 1% ethanolic potassium hydroxide solution, and by allowing an interval of more or less than 10 minutes between addition of water and ethanol. The color fades after 5 minutes. Chloroform, carbon tetrachloride, trichloroethanol, urochloralic acid, chloral hydrate, and trichloroacetic acid do not interfere.

Procedure—*By pyridine.* To a 1-ml. sample containing 0.00007-0.0005 mg. of trichloroethylene, add 5 ml. of pyridine and 0.35 ml. of 1% ethanolic potassium hydroxide solution. After 5 minutes, add 1 ml. of water and let stand for 10 minutes. Clarify the pink turbid solution by addition of 3 ml. of 95% ethanol and read within 5 minutes at 545 mμ.

TOTAL TRICHLORO COMPOUNDS

The determination of total trichloro compounds in urine includes trichloroethanol, urochloralic acid, trichloroacetic acid, and trichloroacetaldehyde. Since urochloralic acid and free trichloroethanol give positive reactions with pyridine and alkali only after oxidation, a chromic oxide-nitric acid oxidizing solution is added. Dichromate-sulfuric acid oxidizing solution either fails to give a quantitative oxidation of trichloroethanol or interferes with the reaction with pyridine and alkali. At least 20 hours at 37° is required for complete oxidation with the chromic oxide-nitric acid reagent. The absorbance produced by trichloroacetic acid after oxidation is greater than that obtained from an equimolar amount of trichloroacetic acid determined from a nonoxidative procedure.[11]

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[10] D. Bonilauri and C. Carola, *Olii minerali, grassi e saponi, colori e vernici* **29**, 35-9 (1952); T. A. Seto and M. O. Schultze, *Anal. Chem.* **28**, 1625-9 (1956).

Trichloroethanol forms a yellow band with a maximum at 440 mμ. Trichloroacetic acid is determined at 370 mμ by heating with alkali before addition of the pyridine to ensure the destruction of trichloroethanol and chloral hydrate. Chloral hydrate is determined by difference. The absorptions of trichloroacetic acid and chloral hydrate are additive; those of chloral hydrate and trichloroethanol are not.

Sample—*Total trichloro compounds. Urine.* Incubate a 1-ml. sample with 1 ml. of 5% chromium trioxide solution in 1:1 nitric acid at 37° for 20 hours. Cool in ice and carefully add 5 ml. of 21% potassium hydroxide solution with cooling. Mix, cool for 5 minutes, and add 10 ml. of pyridine. Insert an air condenser and heat without agitation at 64-65° for 50 minutes. Cool in ice for 5 minutes. Clarify a 5-ml. aliquot of the pyridine layer by addition of 1 ml. of water and read within 20 minutes at 530 mμ.

Simultaneous determination of trichloroacetic acid, trichloroethanol, and chloral hydrate with pyridine. Add 5 ml. of pyridine to 1 ml. of a solution containing 0.02-0.2 micromole of chloral hydrate or trichloroacetic acid and 0.1-0.8 micromole of trichloroethanol. Mix, add 2 ml. of 56% potassium hydroxide solution, and mix. Heat at 100° for 4 minutes and cool in ice for 3-5 minutes. Pipet 3 ml. of the pyridine layer into 0.5 ml. of water and read at 370 mμ and 440 mμ.

For trichloroacetic acid determination, mix 1 ml. of sample with 2 ml. of 56% potassium hydroxide solution and heat at 100° for 2 minutes. Cool in ice for 2-3 minutes and add 5 ml. of pyridine. Mix and follow the procedure for chloral hydrate, trichloroacetic acid and trichloroethanol starting at "Heat at 100° for 4 minutes. . . ." Read at 370 mμ against 3 ml. of pyridine and 0.5 ml. of water.

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[11] T. A. Seto and M. O. Schultze, *Anal. Chem.* **28**, 1625-9 (1956); P. J. Friedman and J. R. Cooper, *Ibid.* **30**, 1674-6 (1958).

Calculate as follows:

$$\mathrm{TCE}^x = \mathrm{TCE}^s \frac{\mathrm{TCE}^x\ 440A}{\mathrm{TCE}^s\ 440A}$$

$$\mathrm{TCA}^x = \mathrm{TCA}^s \frac{\mathrm{TCA}^x\ 370B}{\mathrm{TCA}^s\ 370B}$$

$$\mathrm{CH}^x = \mathrm{CH}^s \frac{\mathrm{CH}^x\ 370A}{\mathrm{CH}^s\ 370A}$$

in which

TCA^s = concentration of trichloroacetic acid in a standard solution
TCA^x = concentration of trichloroacetic acid in an unknown solution
TCA_{370A} = absorbance of a trichloroacetic acid sample in the first part of the procedure
TCA_{370B} = absorbance of a trichloracetic acid sample in the second part of the procedure.

Corresponding superscripts apply to trichloroethanol (TCE) and to chloral hydrate (CH).

PERCHLOROETHYLENE

Perchloroethylene produces a sensitive reproducible color after refluxing with pyridine and aniline followed by refluxing with sodium methylate.[12] The reaction is not specific for perchloroethylene as other halogen compounds react. The accuracy of the method is about ±1 ppm. The color fades and reaches a stable intensity after 90 minutes.

Procedure—*Wheat.* Assemble the apparatus for digestion and aeration as shown in Figure 23. Pack the absorption tube A with activated carbon to remove halogenated compounds in the air. The digestion flask B is heated by a mantle with a Variac control. Maintain the temperature of the condenser C at 65° by means of a flame-heated 15-foot coil of ¼-inch copper tubing connected directly to the cold water tap. Tube D is a condensate trap. Bulbs E and F, sulfuric acid scrubbers, remove water vapor and unwanted materials which distil over from the sample. Their presence decreases the color intensity of the reaction. Bulb G is a mist trap and H and J are pyridine absorbers.

Add 7 ml. of pyridine to the first absorber and 3 ml. to the second. Add 5 ml. of concentrated sulfuric acid to each sulfuric acid scrubber. With the train assembled and 65° water flowing through the condenser,

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[12] J. H. Brumbaugh and D. E. Stallard, *J. Agr. Food Chem.* 6, 465-8 (1958).

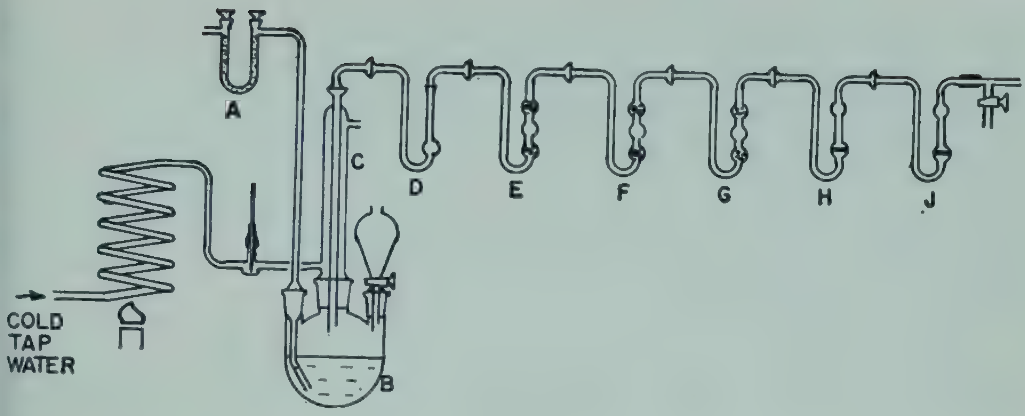

FIG. 23

Apparatus for digestion of samples and aeration of perchloroethylene
A. Activated carbon packed U-tube; B. 500-ml. three-necked, round-bottomed flask, standard taper joints; C. Hot water condenser; D. Condensate trap, 15-mm. tube, 25-mm. outside diameter tube; E,F. Sulfuric acid scrubbers, 25-mm. outside diameter bulbs packed with beads; G. Mist trap; H,J. Pyridine absorbers for perchloroethylene, 25-mm. outside diameter bulb with a medium porosity frit.

add 200 ml. of 1:239 sulfuric acid and an 80-gram sample to the digestion flask and stopper. Maintain a rate of sweep of about 150 ml. of air per minute throughout the system by means of a bleed control on the line going to the aspirator. Adjust the current applied to the heating mantle so that the suspension will boil in 10-15 minutes. Continue heating and sweeping for an additional 45 minutes. Stop the air sweep by venting the bleed line and remove the plug from the neck of the flask. Remove the contents of the first pyridine absorber and wash the absorber with the contents of the second absorber. Rinse both absorbers with 5 ml. of pyridine. To the combined pyridine solutions, add 5 ml. of colorless aniline and reflux for 15 minutes. Add 0.2 ml. of 5% sodium hydroxide solution in methanol without interrupting the heating. After 1 hour of total refluxing, cool under water and dilute the solution to 50 ml. with methanol. Read 90 minutes after the end of the refluxing at 530 mμ against water.

CARBON TETRACHLORIDE

In hot alkaline solution pyridine forms a purplish-red color with carbon tetrachloride (Vol. IV, pp. 513-4). The reaction is one which in various modifications is applied to chloroform, trichloroethylene, chloral hydrate, trichloroacetic acid, and trichloroethanol. Here it is applied to grain in which the presence of interferences is improbable. Samples of wheat or other grain are refluxed with sulfuric acid and phosphotungstic acid, which aid in controlling foaming and in breaking down the kernels. The carbon tetrachloride is then swept out by a gentle air current, absorbed in acetone, and developed. The sensitivity of the method is about 1 ppm.[13]

Carbon tetrachloride in air is absorbed in pyridine and acetone.[14] Copper sulfate in alkaline solution containing thymol is another reagent for carbon tetrachloride.[15]

Samples—*Wheat.* The requisite apparatus is shown in Figure 24. This consists of a 2-neck 1-liter round-bottom flask for aeration, an 8-inch condenser fitted in one neck of the flask and connected to 2 Peligot tube traps in series which are immersed in an ice bath during operation, an air-inlet tube fitted into the second neck of the flask and extending to

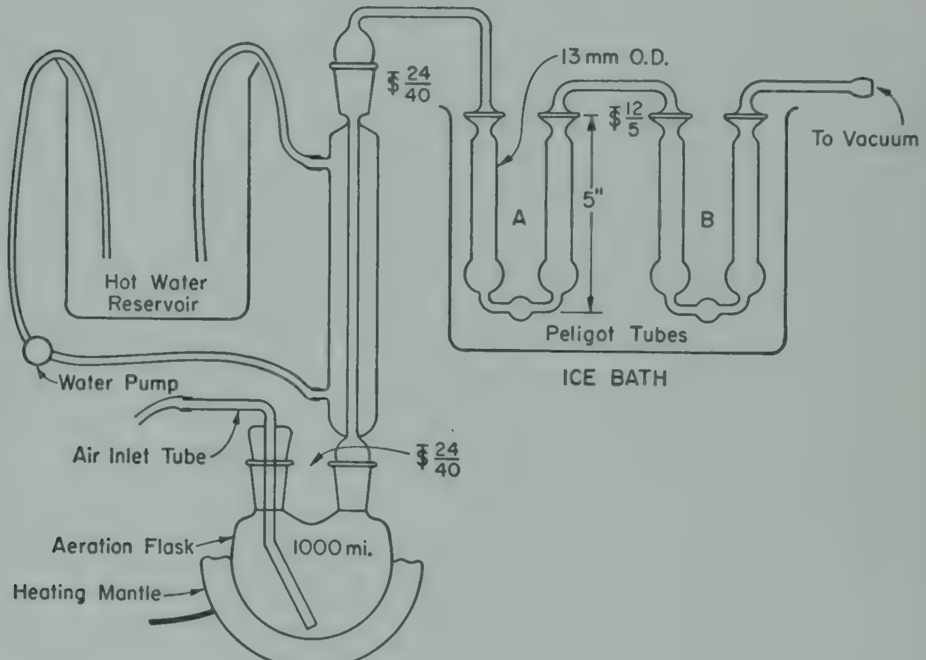
13 mm O.D.
24/40
12/5
5"
A
B
To Vacuum
Hot Water Reservoir
Peligot Tubes
ICE BATH
Water Pump
Air Inlet Tube
24/40
Aeration Flask
1000 ml.
Heating Mantle

FIG. 24

Apparatus for isolation of carbon tetrachloride from grain within 1 cm. of the bottom, a large Mariotte flask of at least 1-liter capacity to serve as a source of vacuum during aspiration, a reservoir containing water maintained at 55-56° which is circulated through the condenser by a small pump, and a heating mantle with rheostat control for heating the aeration flask.

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[13] L. L. Ramsey, *J. Assoc. Offic. Agr. Chemists* **40**, 175-80 (1957).

[14] A. A. Belyakov, *Zavodskaya Lab.* **23**, 161-2 (1957).

[15] P. Blanc, Godrfain, and R. Lescure, *Chim. anal.* **41**, 54-5 (1959).

Connect the isolation apparatus except for the air-inlet tube and the Peligot tubes. Lubricate all fittings with glycerine. Add 4 ml. of acetone to each of the two Peligot tubes, connect them in series to the condenser, and place them in an ice bath. Add 530 ml. of water, 60 ml. of 1:35 sulfuric acid, and 10 ml. of 20% phosphotungstic acid, together with a small amount of DC Antifoam A or 0.1 ml. of DC Emulsion AF to the aeration flask. Immediately add a 100-gram sample to the aeration flask. Fit the air-inlet tube into the flask and close it with a pinch clamp on the rubber tube extension. Start the water pump and the heating mantle. When the mixture boils vigorously, reduce the mantle heat and connect Peligot tube B to the aspirator bottle which has been previously adjusted to draw air through the apparatus at a rate of 25-30 ml. per minute. Lessen the pinch clamp on the air inlet tube to allow the air to be drawn in, being careful to prevent pressure in the aeration flask from forcing the boiling suspension out through the air inlet tube. Adjust the rheostat controlling the heating mantle to maintain vigorous boiling but not so vigorous as to cause large droplets of water to condense in the hot condenser-Peligot connector. Allow the aspiration to proceed for 1 hour.

Stop the operation and pour the acetone from the first Peligot tube through a funnel. Wash the first Peligot tube with the contents of the second Peligot tube and add to the first acetone solution. Rinse the second Peligot tube with 1.5 ml. of acetone and use this rinsing for the first tube. Add to the acetone solutions. Repeat the rinsing with another 1.5-ml. portion of acetone. Dilute the combined acetone solutions to 10 ml. and mix. Take an aliquot which will give an absorbance reading of 0.2-0.5. Develop with pyridine.

Bran and germ. Follow the procedure for wheat, using a 50-gram sample.

Flour and shorts. Add a 100-gram sample in small portions with stirring to 150 ml. of water to form a smooth paste. Add with stirring 330 ml. of water, 60 ml. of 1:35 sulfuric acid, and 10 ml. of 20% phosphotungstic acid. Follow the procedure for wheat, using this mixture in the aeration flask. Wash the beaker containing the sample with two 25-ml. portions of water and add to the aeration flask.

Procedure—*By pyridine.* As a color reagent, add 0.48 ml. of 15% sodium hydroxide solution to 40 ml. of water and mix. Add 100 ml. of colorless pyridine, obtained by distillation over potassium hydroxide, and mix thoroughly. Dilute an aliquot of sample containing 0.2-1 mg. of carbon tetrachloride to 5 ml. with acetone. Add 10 ml. of reagent, stopper, and mix. Remove the stopper and heat at 70° for exactly 15 minutes, maintaining the water level in the bath above the level of the solution. Cool in water at room temperature for 5 minutes and dilute to 16 ml. with water. Stopper and mix. Read at 530 mμ against water.

Air. Aspirate a sample through two tubes containing 3 ml. of a 1:1 mixture of pyridine and acetone at a rate of 15 liters per hour. To a mixture of 1 ml. from each tube add 3 ml. of a solution prepared from 100 ml. of pyridine and 28 ml. of water. Add 0.1 ml. of 0.4% sodium hydroxide solution and mix. Heat for 20 minutes at 85-7°, cool, and add 0.4 ml. of glacial acetic acid and 3-4 drops of aniline. Dilute to 4 ml. and read after 10 minutes at 496 mμ.

By thymol and copper sulfate. Prepare a reagent containing 100 ml. of 30% sodium hydroxide and 10 ml. of 5% copper sulfate solution. Add 1 ml. of 95% ethanol solution containing 40-320 ppm. carbon tetrachloride to a mixture of 2 ml. of reagent and 2 ml. of 5% thymol solution in 95% ethanol. Reflux at 100° for 5 minutes, cool rapidly, and read.

CHLORAL HYDRATE

In the determination of chloral hydrate with pyridine is hot alkaline solution (Vol. III, p. 516), dichloroacetaldehyde, hydrochloric acid and ethanol do not interfere.[16] The technic for total trichloro compounds by this reaction, page 483, permits determination of chloral hydrate in the presence of trichloroethanol and trichloroacetic acid.

Procedure—*By pyridine.* Add a 5-ml. sample containing 0.5-5 mg. of chloral hydrate to a mixture of 5 ml. of 40% sodium hydroxide solution, 10 ml. of pyridine, and 2 ml. of acetone. Heat at 100° for 5 minutes and cool under water for 1-2 minutes. Dilute the pyridine layer to 20 ml. with acetone and read at 530 mμ.

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[16] O. P. Malhotra and V. D. Anand, *J. Indian Chem. Soc.* 34, 501-4 (1957); *Z. Anal. Chem.* 160, 10-4 (1958).

CHLOROBUTANOL, 1,1,1-TRICHLORO-2-METHYL-2-PROPANOL

Chlorobutanol reacts with hydroxylamine in alkaline solution to produce the corresponding hydroxamic acid which forms a colored complex with ferric ion.[17] The color fades at the rate of 5% per hour. Similar colored complexes are obtained with chloral and bromal, although higher temperatures are required.

Esters, acid chlorides, imides, and acid anhydrides which form hydroxamates interfere. Ions which complex with the ferric ion, such as tartarate and citrate, as well as ions which precipitate ferric iron, such as phosphate and phthalate, interfere. A standard must be run with each series because of the variation in absorbance values with different runs. Beer's law is followed for 0.1-0.8 mg. of chlorobutanol per ml.

Procedure—*Parental drug preparations.* In the presence of nonvolatile interferences, add 1 drop of concentrated sulfuric acid to the sample and steam distil for 6 minutes. Rinse down the condenser with water and add the rinsing to the distillate. Dilute the distillate so that 1 ml. contains 0.1-0.6 mg. of chlorobutanol. In the absence of nonvolatile interferences, dilute a sample so that 1 ml. contains 0.1-0.6 mg. of chlorobutanol.

To a 4-ml. aliquot containing 0.4-2.4 mg. of chlorobutanol, add 1 ml. of 7% hydroxylamine hydrochloride solution and 1 ml. of 14% sodium hydroxide solution, swirling after each addition. Let stand at room temperature for 20 minutes. Add 1 ml. of 3:7 hydrochloric acid and swirl Add 1 ml. of 8% ferric chloride hexahydrate solution in 1:110 hydrochloric acid and swirl. Let stand for 5 minutes, shake vigorously, and loosen stoppers to allow gas to escape. Alternatively, bubble a fine stream of air through the solution for 30-60 seconds. Read against a reagent blank at 500 mμ.

EPICHLORHYDRIN

Epichlorhydrin is oxidized with periodic acid to produce formaldehyde. This forms 3,5-diacetyl-1,4-dihydrolutidine, a yellow compound, by reaction with ammonia and acetylacetone.[18] Sodium arsenite eliminates interference from excess periodic acid in the development of the formaldehyde color. Compounds which contain or may be degraded to give terminal vicinal hydroxyl groups, such as ethylene oxide or ethylene glycol, interfere.

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[17] C. R. Rehm and W. J. Mader, *J. Am. Pharm. Assoc.* 46, 621-3 (1957).

[18] J. W. Daniel and J. C. Gage, *Analyst* **81,** 594-8 (1956).

Procedure—*Air.* Draw a 2-liter sample through 8 ml. of water at a rate of approximately 0.5 liter per minute. As an acetylacetone reagent, dissolve 25 grams of ammonium acetate, 3 ml. of glacial acetic acid, and 0.2 ml. of redistilled acetylacetone in water and dilute to 100 ml.

To a 10-ml. sample containing up to 0.1 mg. of epichlorhydrin, add 1 ml. of 1.92% periodic acid solution. Stopper loosely and heat at 100° for 20 minutes. Add 2 ml. of 6.5% sodium arsenite solution, mix, and add 2 ml. of acetylacetone reagent. Stopper and heat for 3 minutes. Cool and dilute to 15 ml. Read at 412 mμ against a reagent blank.

ALKYLCHLOROSILANE

For determination in air, alkylchlorosilane is absorbed in concentrated sulfuric acid. Thereafter it is decomposed to silica and converted to sodium silicate. The silicate is then determined as molybdenum blue[19] (Vol. IIIA, pp. 581-6).

Procedure—*Air.* Draw a 5-10 liter sample at a rate not exceeding 5 liters per hour through two microabsorbers each containing 5 ml. of concentrated sulfuric acid. Remove the contents of the absorbers and heat until the sulfuric acid fumes are driven off. Add 0.1 gram of sodium potassium carbonate and 3 ml. of water to the residue. Mix, evaporate to dryness, and fuse. Leach 3 times with 5 ml. portions of hot water. Carefully acidify to litmus using 1:35 sulfuric acid and heat to expel carbon dioxide. Dilute to 20 ml. with water.

To a 4-ml. aliquot, add 0.1 ml. of 10% ammonium molybdate solution in 8:92 sulfuric acid. Shake and, after 5 minutes, add with shaking 1 ml. of 5% tartaric acid and 0.1 ml. of 1% ascorbic acid. Read at 815 mμ.

CHLOROCRESOL

A color forms between chlorocresol and a mercuric reagent in sulfuric acid.[20] A similar color is developed by quinosol, 8-quinolinol sulfate, and the reagent.

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[19] E. A. Peregud and N. P. Kozlova, *Zhur. Anal. Khim.* **9,** 47-50 (1954).

[20] W. Filz, *Osterr. Chem.-Ztg.* **55,** 107-8 (1954).

Procedure—To prepare the reagent, dissolve 70 ml. of concentrated sulfuric acid, 70 grams of sodium sulfate, 75 grams of mercuric sulfate, and 55 grams of mercuric chloride in 800 ml. of water and dilute to 1 liter. Treat a 2-ml. sample containing 0.1% of chlorocresol with 2 ml. of reagent. Heat at 100° for 2 minutes. Filter, add 0.1 ml. of 6% sodium nitrate solution, and read.

TETRACHLOROHYDROQUINONE

Tetrachlorohydroquinone produces a wine-red color with triethylamine when tetrachlorohydroquinone is added to a triethylamine-benzoyl peroxide mixture. The color is due to chloranil, the oxidized form of tetrachlorohydroquinone.[21] The order of addition of reagents is of prime importance. The tetrachlorohydroquinone added to an ethyl acetate solution of the reagent and oxidizing agent which has been standing 20-30 minutes produces a green-blue color with a maximum absorption at 610 mμ. However, when the triethylamine reagent is added to a solution of the tetrachlorohydroquinone and oxidizing agent a wine-red color develops with a maximum absorption at 530 mμ. The green-blue color is more satisfactory for colorimetric determination of tetrachlorohydroquinone since there is less interferences at 610 mμ from substances such as tannins which are extracted with the tetrachlorohydroquinones.

As the color is effected by the solvent, ethyl acetate is preferred for this method. Approximately the same result is obtained in benzene or tolune, but the color development is slower. A yellow or yellow-green color develops in ethanol and acetone, while in chloroform a purple-red or orange-red color develops, depending on the order of addition of reagents. Small amounts of water do not interfere. The color is sensitive to sunlight. Quinone and hydroquinone give a faint purple color which develops slowly and does not interfere unless the quinone and hydroquinone are present in large amounts. Phenol, chlorinated phenols, tanning agents, or leather components do not interfere. Since this method is actually a reaction of chloranil, tetrachlorohydroquinone cannot be distinguished from chloranil by this method. Beer's law is followed for 0.05-0.3 mg. per 50 ml.

Procedure—*Leather*. Extract a 2.5-gram ground sample in a Soxhlet-type apparatus with anhydrous or 90% ethyl acetate solution. Dilute the combined extracts to 100 ml. with ethyl acetate if the amount of tetrachlorohydroquinone does not exceed 1.2%. If the percentage in the sample is higher, dilute further.

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[21] Sverre Dahl, *Anal. Chem.* **25,** 1724-7 (1953).

Combine 5 ml. of 2% benzoyl peroxide solution in ethyl acetate and 2 ml. of triethylamine and let stand at room temperature for 30 minutes. Add a 1-5 ml. sample containing 0.05-0.3 mg. of tetrachlorohydroquinone in ethyl acetate and let stand protected from sunlight at room temperature for 90 minutes. Dilute to 50 ml. with ethyl acetate and read within 30 minutes against ethyl acetate at 610 mμ.

BENZALKONIUM CHLORIDE

A turbimetric assay of benzalkonium chloride is based on the reaction of quaternary ammonium compounds to form insoluble salts with anionic substances.[22] Sodium salicylate is the precipitating agent and methylcellulose is added for stability of the suspension. An excess of sodium salicylate, sodium nitrite, and phosphate buffers have no influence. Ethanol and isopropanol decrease the optical density at high concentrations. Benzethonium chloride yields a precipitate by this method similar to that of benzalkonium chloride while cetyl pyridenium chloride or cetyl trimethyl ammonium bromide do not.

Procedure—Dilute a sample to a concentration of 0.0025%-0.05%. To a 5-ml. aliquot, add 5 ml. of 0.5% sodium salicylate solution containing 0.5% of methylcellulose. After 10 minutes determine the loss of transmitted light at 450 mμ against a reagent blank.

CHLOROPICRIN, NITROCHLOROFORM

In hot alcoholic sodium peroxide solution, chloropicrin decomposes to form sodium carbonate, sodium chloride, sodium nitrite, and water. Chloropicrin is then estimated by the concentration of liberated nitrite ion usng sulfanilic acid and a *N*-(1-naphthyl) ethylenediamine dihydrochloride coupling reagent.[23] Beer's law is followed.

Pyridine is converted by chloropicrin to glutaconaldehyde which forms a color when condensed with phloroglucinol.[24] A similar method employs dimedone (5,5-dimethyl-1,3-cyclohexanedione) in place of phloroglucinol.

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[22] R. A. Post and O. H. Miller, *Am. J. Hosp. Pharm.* 15, 150-2 (1958).

[23] L. Feinsilver and F. W. Oberst, *Anal. Chem.* 25, 820-1 (1953).

[24] R. M. Fourneur and M. Person, *Chim. anal.* 39, 155-8, 263-6 (1957).

Sample—*Air.* Bubble a 2-liter sample through 5 ml. of methyl Cellosolve in 15 minutes and develop with pyridine and dimedone.

Procedure—*As the nitrite ion.* To a solution containing 0.0122-0.0915 mg. of chloropicrin per ml. in isopropanol add 10 ml. of 4.5% sodium peroxide solution and dilute with isopropanol to 20 ml. Reflux for 30 minutes and cool. Add one drop of phenolphthalein reagent and adjust the pH with concentrated hydrochloric acid until the pink color just disappears. Add 1 ml. of 0.8% sulfanilic acid in 30% acetic acid solution, mix thoroughly, and let stand for 1 minute. Immediately add 1 ml. of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride solution, mix, and dilute to 25 ml. with water. Read at 540 mμ after 15 minutes.

By pyridine and phloroglucinol. Extract a sample containing 0.01-0.04 mg. of chloropicrin in aqueous solution with hexane. Prepare a reagent by mixing 50 ml. of pyridine, 50 ml. of methanol, 0.6 gram of potassium cyanide, and 0.1 gram of phloroglucinol. To a 2-ml. portion of the extract, add 5 ml. of reagent. Mix and read after 5 minutes at 535 mμ.

By pyridine and dimedone. To 5 ml. of methyl Cellosolve containing 0.0004-0.006 mg. of chloropicrin per ml., add 2 ml. of a reagent prepared from 30 ml. of pyridine, 70 ml. of methanol, 0.8 gram of dimedone, and 0.4 gram of potassium cyanide. Heat for 5 minutes, cool, and read at 490 mμ.

Terephthaloyl and Isophthaloyl Chlorides

Terephthaloyl and isophthaloyl chlorides are separated from their corresponding free acids by washing the sample with isooctane. The free acids are insoluble in the isooctane. They are then read directly. The maximum absorbance of isophthaloyl chloride is at 227 mμ. Terephthaloyl chloride shows absorbance peaks at 254 and 263 mμ.[25] If phthaloyl chloride is present, a third wave length must be used and three equations derived. Thionyl chloride does not interfere at the wave lengths used.

Procedure—To a 0.4-0.6 gram sample add 3-5 ml. of isooctane and slurry the mixture. Very slowly and carefully draw the liquid through a sintered glass filter. Repeat the slurrying and filtration 7-8 times. Wash the underside of the crucible and the stem of the funnel with a fine stream of solvent and dilute the filtrate to 50 ml. Read at 227 and 263 mμ.

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[25] Ernest J. Breda, *Anal. Chem.* 30, 2020-2 (1958).

Calculate the acid chlorides as follows:

mg. of isophthaloyl chloride per 50 ml. of final solution

$$= 0.2860A_{227} - 0.0305A_{263}$$

mg. of terephthaloyl chloride per 50 ml. of final solution

$$= 0.4720A_{263} - 0.0049A_{227}.$$

3-CHLOROPROMAZINE, LARGACTIL

Chloropromazine extracted into dilute aqueous sulfuric acid is read directly.[26] Hydrolysis permits determination of bound promazine by the same technic. A mixture of chloropromazine and its sulfoxide is indicated by absorption at 240 mμ and/or 255 mμ, and 274 mμ and 305 mμ. The secondary peak for chloropromazine sulfoxide occurs at 274 mμ, the maximum peak for chloropromazine occurs at 255 mμ,[27] and the common peak for both compounds is 305 mμ. The concentration of chloropromazine in the presence of its sulfoxide is determined by a background cancellation method.

A red color is produced by the oxidation of 3-chloropromazine with sulfuric acid and ferric ion.[28] Beer's law is followed up to 0.025 mg. per ml. and with a slight deviation of 5% up to double that amount.

Phosphoric and iodic acids are also a reagent for development of 3-chloropromazine.[29] To remove promethazine which interferes, an ionophoretic separation in acetone buffer at pH 4 is required. The chloropromazine is then eluted from the paper with 1:3500 sulfuric acid and developed.

Chloropromazine produces a red color in nearly concentrated sulfuric acid.[30] The procedure is also modified to use nitric acid.[31] The color is the same. Methyl orange yields a red with the maximum absorption at 550 mμ.[32]

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[26] T. L. Flanagan, T. H. Lin, W. J. Novick, I. M. Rondish, C. A. Bocher, and E. J. Van Loon, *J. Med. Pharm. Chem.* **1**, 263-73 (1959).

[27] Carlos R. Gil Salas, *Anales fac. farm. y bioquím. Univ. nacl. mayor San Marsos (Lima)* **8**, 500-9 (1957).

[28] H. Leach and W. R. C. Crimmin, *J. Clin. Pathol.* **9**, 164-5 (1956).

[29] A. Calo, A. Mariani, and O. M. Marelli, *Svensk. Farm. Tidskr.* **60**, 842-7 (1956).

[30] P. Dubost and S. Pascal, *Ann. pharm. franc.* **11**, 615-19 (1953).

[31] C. Citterio, *Lav. psichiat.* **20**, 189-93 (1957).

[32] S. Yasuda and K. Aoki, *Osaka Shiritsu Daigaku Igaku Zasshi* **6**, 741-6 (1957).

Chloropromazine is read fluorimetrically at 480 mμ and pH 11 when activated at 350 mμ.[33] Chloropromazine sulfoxide is read at 400 mμ at pH 7 when activated at 335 mμ.

Procedure—*Direct reading. Urine. Free chloropromazine and chloropromazine sulfoxide.* To a 5-ml. sample containing a minimum of 0.005 mg. of chloropromazine and its sulfoxide, add 5 ml. of water and adjust the pH to 12 with 40% sodium hydroxide solution. Add 25 ml. of ether and shake vigorously for 2 minutes. Separate and re-extract the aqueous phase 4 times with 25-ml. portions of ether.

To the combined ether extracts, add 1 ml. of 0.4% sodium hydroxide solution and shake vigorously for 1 minute. Separate and add the alkaline layer to the remaining layers from the urine extraction. Reserve this to determine bound chloropromazine and its sulfoxide by the technic following this. Shake the ether layer with 2 ml. of water vigorously for 1 minute. Allow the layers to separate for 5 minutes, draw off the water layer, and discard.

Add 5 ml. of 1:35 sulfuric acid and shake vigorously for 3 minutes. Allow the layers to separate for 10 minutes. If the acid layer is not clear, centrifuge for 3 minutes. If the concentration of chloropromazine is high, repeat the acid extraction. If the concentration of chloropromazine in the extract is high, dilute with 1:35 sulfuric acid.

If only chloropromazine is present read at 255 mμ. If the sulfoxide is also present read at 236 mμ, 255 mμ, and 265 mμ. Then determine the chloropromazine by drawing a diagonal line connecting the points at 236 mμ and 265 mμ. Read the absorbance by measuring from the point at 255 mμ down to the corresponding point on the diagonal line. Calculate the sulfoxide by subtraction of the absorbance at 315 mμ from that at 270 mμ.

Bound chloropromazine and chloropromazine sulfoxide. Warm the aqueous extract reserved from the determination of free chloropromazine in urine to remove ether, shaking frequently. Add sodium hydroxide pellets to bring the sodium hydroxide concentration to 3%. Heat at 100° for 1 hour and cool to room temperature. Extract with five 40-ml. portions of ether. Combine the ether extracts and follow the procedure for free chloropromazine in urine starting with "To the combined ether extracts, add 1 ml. of 0.4% sodium hydroxide solution. . . ."

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[33] S. Udenfriend, D. E. Duggan, B. M. Vasta, and B. B. Brodie, *J. Pharmacol. Exptl. Therap.* **120, 26-32 (1957).**

Free and bound chloropromazine and chloropromazine sulfoxide. Add sodium hydroxide pellets to the sample to make the sodium hydroxide concentration 3%. Heat at 100° for 1 hour, cool, and extract with five 40-ml. portions of ether. Follow the procedure for free chloropromazine in urine starting "To the combined ether extracts, add 1 ml. of 0.4% sodium hydroxide solution. . . ."

By sulfuric acid and ferric ion. Urine. To a 20-ml. sample, add 1 ml. of 4% sodium hydroxide solution and extract with four 20-ml. portions of ether. Wash the combined extracts with 10 ml. of 0.8% sodium hydroxide solution and 10 ml. of water. If the chloropromazine is below 1 mg. per liter, extract from the ether extracts with 6 ml. of 1:350 sulfuric acid. Otherwise extract with 10 ml. of 1:350 sulfuric acid. Separate the acid extract after 5 minutes. Warm the acid extract and aerate to remove traces of ether.

Treat a 4-ml. aliquot containing 0.005-0.1 mg. of 3-chloropromazine with 2 ml. of 1:1 sulfuric acid and mix well. Add 0.2 ml. of 2% ferric nitrate solution in 1:35 sulfuric acid and read at 530 mμ against water.

Blood. Free chloropromazine. Heat a 5-ml. oxalated sample with 5 ml. of 50% potassium hydroxide solution at 100° for 2 minutes with frequent shaking. Cool and add 10 ml. of water. Shake with 20 ml. of ether. Centrifuge and repeat the ether extraction three times. Wash the combined ether extracts following the procedure for urine starting with "Wash the combined extracts with 10 ml. of 0.8% sodium hydroxide solution. . . ."

Total chloropromazine. Heat a 5-ml. sample with 5 ml. of concentrated hydrochloric acid at 100° for 5 minutes. Cool and cautiously make the solution alkaline. Follow the procedure for free chloropromazine in blood starting at "Shake with 20 ml. of ether."

By phosphoric and iodic acids. To 1.5 ml. of a solution of chloropromazine hydrochloride add 2.5 ml. of 85% phosphoric acid and 1 ml. of 10% iodic acid solution. Read the red color at 526 mμ.

In strong sulfuric acid. Urine and blood. A suitable urine sample is 20 ml. A suitable blood sample is 5 ml. diluted to 20 ml. with water. Make the solution alkaline and extract 3-4 times with 20 ml. portions of ether. Wash the combined extracts with 0.8% sodium hydroxide solution. Extract the free chloropromazine from the ether with 10 ml. of 1:350

sulfuric acid. Concentrate the extract in sulfuric acid to 2.5 ml. by warming and applying a vacuum if necessary. Add 2.5 ml. of concentrated sulfuric acid. Read the carmine-red color against a free chloropromazine standard. To determine the total free and conjugated chloropromazine, hydrolyze the sample and follow the above procedure.

By nitric acid. Blood or cerobrospinal fluid. To a 5-ml. oxalated blood sample, add 4 ml. of concentrated hydrochloric acid and 1 ml. of water. Heat at 100° for 5 minutes, cool, dilute to 20 ml., and neutralize with 50% sodium hydroxide solution. Extract with three 20-ml. portions of ether for 5 minutes each. Wash the combined extracts twice with 10 ml. of 2% sodium hydroxide solution and wash once with 10 ml. of water. Shake with 6 ml. of 1:350 sulfuric acid. Heat the sulfuric acid phase to 55-60° to expel ether. To a 2-ml. portion, add 0.2 ml. of concentrated nitric acid and read at 514 mμ against a water blank.

Urine. Boil a 20-ml. sample with 16 ml. of concentrated hydrochloric acid and 4 ml. of water. Cool and neutralize with 50% sodium hydroxide solution. Add 3 ml. of 20% sodium carbonate solution and follow the procedure for blood above starting at "Extract with three 20-ml. portions of ether. . . ."

CHLORAMPHENICOL

The nitro group of chloramphenicol reacts with dimethylformamide in acetone in the presence of tetraethylammonium hydroxide. The red color formed does not conform to Beer's law in the absence of acetone.[34] An excess of acetone reacts with dimethylformamide in the presence of alkali to give a yellow color which interferes with the determination of chloramphenicol. Color is produced more rapidly by adding a solution of chloramphenicol in dimethylformamide to the dimethylformamide-acetone-tetraethylammonium hydroxide mixture than by adding tetraethylammonium hydroxide to the chloramphenicol in dimethylformamide and acetone. Chloramphenicol is developed as a hydroxamic acid with ferric ion.[35]

Procedure—*By dimethylformamide and tetraethylammonium hydroxide.* To 10 ml. of dimethylformamide, add 0.2 ml. of 0.1% acetone solution in dimethylformamide and 0.1 ml. of 25% tetraethylammonium hydroxide solution. Shake gently and add a sample containing 0.1-0.15 mg. chloramphenicol in dimethylformamide. Shake gently and read after 10 minutes at 520 mμ against a reagent blank.

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[34] F. M. Freeman, *Analyst* **81**, 299-301 (1956).

[35] T. Aihara, H. Machida, and Y. Yoneda, *Yakugaku Zasshi* **77**, 1318-21 (1957).

By hydroxylamine hydrochloride and ferric chloride. Heat a sample containing 50 mg. of chloramphenicol with 30 ml. of water at 80°, cool to room temperature, and dilute to 50 ml. with water. Mix a 3-ml. aliquot with 0.5 ml. of 14% hydroxylamine hydrochloride solution and 0.5 ml. of 10% sodium hydroxide solution. Heat for 10 minutes at 60° and cool to 10-15°. Stir in 0.5 ml. of 1:5 hydrochloric acid and 2 ml. of 1% ferric chloride solution. Read after 5 minutes at 505 mμ.

2,2′-METHYLENEBIS(3,4,6-TRICHLORO)PHENOL, HEXACHLOROPHENE, G-11

Hexachlorophene in alkaline solution shows an absorption maximum at 320 mμ. Soap solutions in alkali show only low general absorption at 320 mμ, and a correction is applicable to take this factor into account.[36] The precision of the method is ±3%. There is no interference by 2,2′-methylene-bis-(4-chlorophenol) and 2,2′-thio-bis-(4,6-dichlorophenol).

A differential spectrophotometric method involves the determination of the absorbance of a dilution of hexachlorophene at alkaline pH against the same concentration in acid pH.[37] The change in absorption represents the change of the hexachlorophene from the molecular form to the ionic form. This procedure eliminates error caused by absorption of the soap base in soap samples. Beer's law is followed.

Procedure—*Soap.* Dissolve a finely shredded 0.05-0.1 gram sample with a minimum of warming in 25 ml. of 0.56% potassium hydroxide solution. Dilute to 100 ml. with 0.56% potassium hydroxide solution. Read at 311.5, 320, and 326 mμ against potassium hydroxide solution. Apply the following correction:

$$E_{\text{corr.}} = 4.787E_{320} - 2.805E_{326} - 1.982E_{311.5}$$

$$\%\ \text{hexachlorophene} = \frac{E_{\text{corr.}} \times 100}{302.5 \times c \times l}$$

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[36] J. W. Lord, I. A. McAdam, and E. B. Jones, *Soap, Perfumery and Cosmetics* **26**, 783-7 (1953).

[37] R. F. Childs and L. M. Parks, *J. Am. Pharm. Assoc.* **45**, 313-6 (1956).

in which 302.5 is the extinction coefficient for pure hexachlorophene, c is the percentage concentration of the solution with respect to soap, and l is the length in cm. of the absorption cell.

Liquid soaps. Differential method. As an acid reagent dilute 18 grams of glacial acetic acid to 1 liter with 90% methanol containing 0.1% by volume of concentrated hydrochloric acid. As an alkaline reagent adjust 90% methanol to pH 8 by addition of 0.4% sodium hydroxide solution.

Dilute a 5-gram sample to 100 ml. with 95% ethanol and mix thoroughly. Dilute a 25-ml. aliquot to 100 ml. with 90% methanol and mix well. Dilute one 15-ml. aliquot to 50 ml. with the acid reagent. Dilute a second 15-ml. aliquot to 50 ml. with the alkaline reagent. Read the sample at pH 3 against the sample at pH 8 at 312 mμ.

HEXACHLOROCYCLOHEXANE, BENZENE HEXACHLORIDE, LINDANE

Benzene hexachloride is determined as *m*-dinitrobenzene after dechlorination by zinc and acetic acid to benzene and nitration (Vol. III, pp. 518-23). Interferences in mushroom samples are removed by sulfonation of a methylene chloride extract.[38]

Benzene hexachloride is collected from air in acetic acid or on an alumina column and the adsorbed lindane is washed off with acetic acid.[39] Since the determination of lindane in the air measures both the vapor and the solid particles, an average of several determinations is necessary for estimation of the total lindane in air. All glass connections should be used as rubber affects the results.

Benzene hexachloride to trichlorobenzene, nitrated to trichlorodinitrobenzene and hydrolyzed with sodium hydroxide to chlorodinitroresorcinol which produces the yellow color for reading.[40] The main color-producing compound is 6-chloro-2,4-dinitroresorcinol, but 2-chloro-4,6-dinitroresorcinol and 5-chloro-2,4-dinitroresorcinol may also contribute to the color. Benzene hexachloride is separated from fats by chromatography through kieselguhr and a mixture of carbon tetrachloride and oleum. As much as 40% of the benzene hexachloride may be lost during the series of reactions. Some loss occurs in the nitration step.

Samples—*Insects.* Grind the sample with anhydrous sodium sulfate until finely subdivided and extract 3 times with a total of 20 ml. of carbon tetrachloride stirring for 4-minute intervals for each extraction. Filter the combined extracts with suction. Mix 3 grams of kieselguhr, 2 ml. of 30% oleum, and enough carbon tetrachloride to produce a stiff slurry. Prepare a column 2 cm. in diameter and 10 cm. long. Add 5 ml. of carbon tetrachloride, followed by the slurry. Tamp lightly until a depth of 4 cm. is attained. When the excess carbon tetrachloride is practically drained off, add the extract of the sample to the column and allow to filter. Wash the column 4 times with 3-4 ml. portions of carbon tetrachloride and finally apply suction for 30 seconds. Develop as 6-chloro-2,4-dinitroresorcinol.

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[38] Irwin Hornstein, *J. Agr. Food Chem.* **3,** 848-9 (1955).

[39] I. Hornstein and W. N. Sullivan, *Anal. Chem.* **25,** 496-8 (1953).

[40] F. R. Bradbury and H. Standen, *J. Sci. Food Agr.* **5,** 252-6 (1954).

Mushrooms. Wash a 100-gram sample, cut into small pieces, and reflux for 3-4 hours with 200 ml. of methylene chloride. Let stand overnight. Decant and filter. Allow any water present to rise to the top and drain off the methylene chloride layer. Concentrate the methylene chloride to approximately 50 ml. Shake vigorously for 2 minutes with 10 ml. of 30% fuming sulfuric acid, allow the layers to separate, and discard the bottom acid layer. To ensure complete sulfonation, repeat the reaction with two 10-ml. portions of 30% fuming sulfuric acid. Cautiously add 25 ml. of cold water to the methylene chloride. Shake thoroughly, allow the layers to separate, and discard the water layer. Wash the methylene chloride with two additional 25-ml. portions of cold water. Evaporate the methylene chloride to 1-2 ml. at 100°, add 120 ml. of glacial acetic acid, and distil 20 ml. of the acid at 130-140° to remove the last traces of volatile solvent. Develop the acetic acid solution as *m*-dinitrobenzene (Vol. III, pp 521-2).

Air. Absorption in acetic acid. Assemble an apparatus consisting of two gas-washing bottles equipped with fritted-glass gas dispersers and a purifying jar, wide-necked at the top and tubular at the bottom. Connect the purifying jar to a calibrated gas meter followed by a diaphragm-type compressor equipped with a bleed valve to regulate the flow through the absorbers.

Fill each gas-washing bottle with 125 ml. of glacial acetic acid and add a 1:1 mixture of 8-mesh soda lime and 8-mesh silicic acid to the purifying jar. Draw the air sample through the gas-washing bottles at a rate of 1 cubic foot per hour until 0.05-0.1 mg. of lindane is collected. The first bottle absorbs most of the lindane. Pass the sample through the purifying jar to remove traces of acetic acid.

Wash the acetic acid from the first bottle into a flask equipped with a 24/40 joint about 150 mm. long to minimize losses of acid. Wash the first bottle with the acetic acid from the second bottle. Concentrate the acid to half its volume to remove interferences of volatile materials such as benzene and develop as *m*-dinitrobenzene (Vol. III, pp. 521-2).

Adsorption on alumina. Prepare an adsorption column 200 mm. long and 25 mm. in diameter with an attached bottom stem about 90 mm. long. Place glass wool at the bottom and add 20 grams of 80-200 mesh alumina. Top with another glass wool plug. Pack the column by applying suction. Connect the tube to the vacuum side of the compressor through a tightly fitted rubber stopper set in a suction flask to act as a holder. Attach a gas meter to the pressure side of the compressor.

Pass an air sample containing 0.05-0.1 mg. of lindane through the meter at rates as high as 0.2 cubic feet per minute by adjusting the bleed valve on the vacuum side. When the outlet of the gas meter is open to atmospheric pressure regardless of the rate of flow, a partial vacuum cannot form inside the gas meter container to cause the sides to buckle.

Wash the column with 150 ml. of glacial acetic acid in small portions to remove lindane. Concentrate the acid to half its volume to remove interferences by volatile materials such as benzene. Develop as *m*-dinitrobenzene (Vol. III, pp. 521-2).

1,1,1-TRICHLORO-2,2-BIS(*p*-CHLOROPHENYL)ETHANE, DDT

The procedure of determining DDT by nitration followed by development with sodium methylate (Vol. III, pp. 535-40) is modified by replacing sodium methylate with sodium methoxide.[41] Interferences are removed by chromatography through Celite, although this procedure lowers recoveries.

Nitrated DDT reacts with isopropylamine in benzene to form an intense yellow color with an absorption peak at 350 mμ.[42] The color is stable in the presence of strong alkalies, but reddens after several hours. The method is sensitive to 0.05 mg. of DDT and the results are reproducible from 0.1-1 mg.

Dilute sodium hydroxide dehydrochlorinates *p,p′*-DDT to the olefin, 2,2-bis(*p*-chlorophenyl)-*l*-dichloroethylene, which absorbs strongly at 250 mμ. The impurity *o,p′*-DDT which occurs in commercial products changes only slightly to its olefin. The method is relatively specific.[43] The method is also applicable with potassium hydroxide.[44] Bis(*p*-chlorophenyl) sulfone and 2-trichlor-*l*-*o*-chlorophenylethyl-*p*-chlorobenzene sulfonate do not interfere. Crude kerosene causes a high blank value. The tempreature of the method should be constant. Beers' law is followed.

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[41] Ben Berck, *Anal. Chem.* **25**, 1253-6 (1953); Cf. Werner Perkon, *Z. anal. Chem.* **134**, 267-73 (1951); G. Brückner, R. Flatow, and M. Rohrlich, *Getreide u. Mehl* **7**, 73-7 (1957).

[42] R. C. Amsden and D. J. Walbridge, *J. Agr. Food Chem.* **2**, 1323-4 (1954).

Samples—*River water.* Acidify a sample to pH 4-5 with dilute sulfuric acid. Extract a 1500-ml. portion with 250 ml. of a 3:1 mixture of peroxide-free ether and *n*-hexane for 10 minutes. Follow with 200 ml. of mixed solvent for 6 minutes and with 150 ml. of mixed solvent for 4 minutes. Wash the separatory funnels with 15-ml. portions of solvent and add to the combined extracts. Drain off any water from the extract and add 10 grams of anhydrous sodium sulfate. Shake gently until the sodium sulfate cakes, then vigorously for 1 minute, and let stand for 2 minutes. Filter half the extract and evaporate to near dryness at 100°. Add the remaining extract and wash the separatory funnel with two 15-ml. portions of solvent. Add the balance of the extract and wash the extraction funnel with 15 ml. and 15 ml. of solvent. Filter the washings into the extract. Evaporate to near dryness at 100° and remove the last traces of solvent with a moderate vacuum and a water aspirator. As a blank use a sample of untreated river water. Follow the procedure by nitration, Vol. III, p. 539. If the water blank exhibits a small absorption at 600 mμ with no brown-yellow color as compared with the reagent blank, then there is no interference present and the residue can be developed in that way.

If the water blank gives an appreciably absorption value then the sample must be cleared of interferences by chromatography. Dissolve the residue in carbon tetrachloride and pass through a chromatograph column following the procedure in Vol. III, p. 538, starting with "Triturate 30 grams of Celite 545 with 9 ml. of 15% fuming sulfuric acid. . . ." Evaporate the carbon tetrachloride and redissolve the residue in acetone. Filter. If the sample contains less than 0.04 mg. of DDT, add a standard amount of 0.01 mg. of DDT in acetone. Evaporate the acetone with an air jet and follow the procedure by nitration, Vol. III, p. 539 with the following modifications. Use 10% potassium hydroxide solution in place of 2% sodium hydroxide solution. Read the color in microcells using 13% sodium methoxide solution to develop the blue color. Use a correction

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[43] B. Herriott, *Science* **104**, 228-30 (1946).

[44] Hector Mazzarri, *Rev. sanidad y assistencia social* (*Venezuela*) **17**, 211-3 (1952).

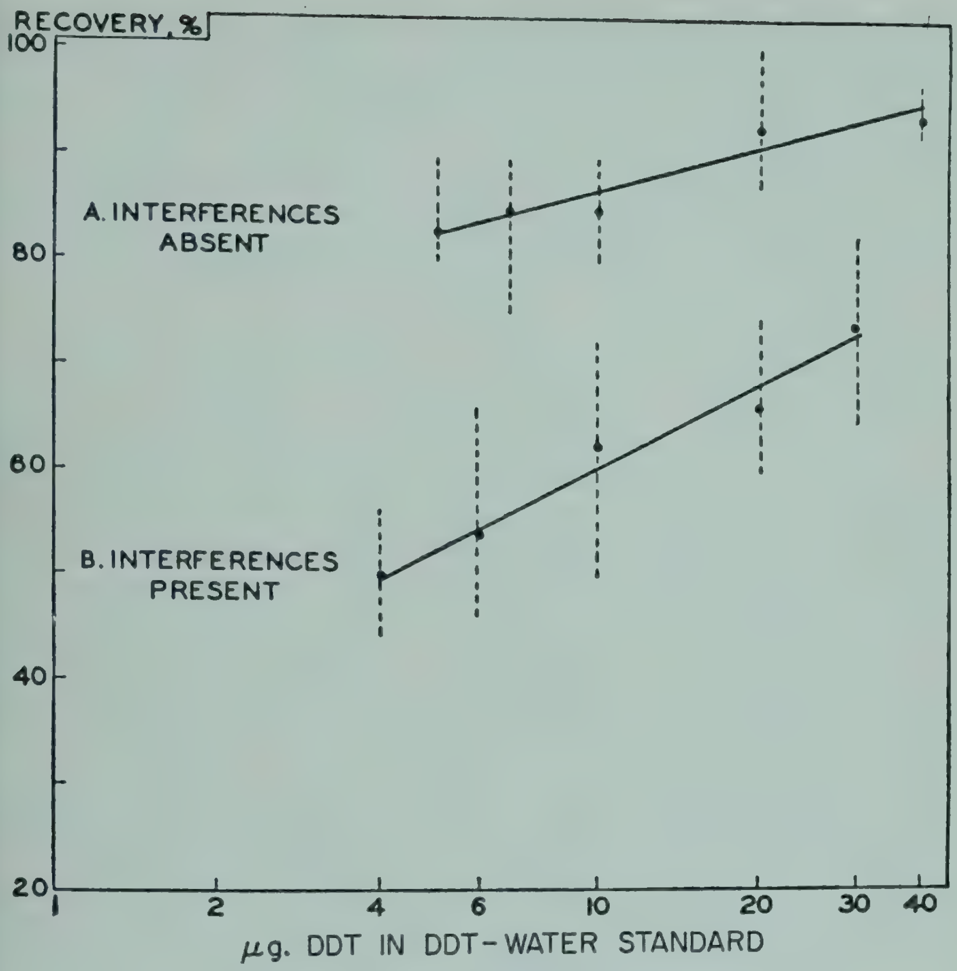

Fig. 25

Correction curve for microdetermination of DDT in river water

curve such as shown in Figure 25 to correct the DDT loads actually found to a theoretical 100% recovery.

River water containing suspended solids. Acidify each gallon of water with 5 ml. of 1:16 sulfuric acid and mix for 30 seconds. Set the samples at a 45° angle and let stand until it appears that no further sedimentation will occur. Siphon out most of the supernatant liquid leaving 2 inches of water above the sediment. Combine the samples, let settle as before, and siphon leaving 0.5 inch of water.

Transfer to evaporating dishes and wash the jars with acetone, using a total of 25 ml. for each rinse. When the samples are sufficiently dry, extract overnight with hexane. Evaporate the solvent and redissolve the residue in carbon tetrachloride. Develop by nitration, Vol. III, p. 539, making the substitutions as described under river water sample starting with "Use 10% potassium hydroxide solution. . . ."

Leaves or slides. Wipe the surface of the sample with a cotton swab washed in ether. Wash the cotton through a funnel with ether and wash the sample with a small quantity of ether. Evaporate the ether at 100° and develop the residue with isopropylamine.

Procedure—*By isopropylamine.* To a sample containing 0.1-1 mg. of DDT, add 3-4 ml. of a 1:1 mixture of fuming nitric acid and concentrated sulfuric acid. Heat at 100°. At 2.5 minutes after brown fumes have begun to evolve cool in water. Wash the mixture into 10 ml. of water using three 2-ml. portions of water. Cool in water.

Shake vigorously with 10 ml. of benzene for 30 seconds. To a 1-ml. portion of the benzene layer add 3 ml. of a 1:3 mixture of isopropylamine and benzene. Shake gently and compare the yellow color with potassium dichromate standards against a blank of benzene and isopropylamine. In the presence of oils and chlorophyll read against a portion of the benzene extraction.

By dehydrochlorination. Heat a 3-ml. sample containing 0.002-0.02 mg. of DDT per ml. at 37° with 0.5 ml. of 2.8% sodium hydroxide solution in 95% ethanol. After 5 minutes, neutralize with 0.1 ml. of 1:3 acetic acid. Read at 250 mμ. Subtract a sample blank in which the acid and alkali are mixed before addition to the sample.

1,1,1-TRICHLORO-2,2-BIS(*p*-METHOXYPHENYL)ETHANE, METHOXYCHLOR, METHOXY DDT

Methoxychlor is dehydrohalogenated with potassium hydroxide to 1,1-dichloro-2,2-bis(*p*-methoxyphenyl)ethylene which is treated with 85% sulfuric acid and read at 555 mμ.[45] A small amount of ferric chloride added to the sulfuric acid reagent increases the rate of color development. Water destroys the color, therefore the color is stable for several days only in the absence of atmospheric moisture.

Extraction from plants is accomplished with benzene or petroleum ether. Dehydrohalogenated methoxychlor is isolated from fats by hot acetone or by acetonitrile.[46] Carotenes and residues which produce char and turbidity are removed by chromatography through magnesia and Celite. If small quantities of animal tissues are analyzed such as 4 grams of fat, 5 grams of kidney, or 10 grams of liver, the hydrolysis and chromatography step will isolate the methoxychlor from the fats and waxes and the extraction with acetonitrile can be eliminated.

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[45] J. D. Fairing and H. Warrington, "Advances in Chemistry No. 1," pp. 260-5, American Chemical Society, 1950.

Methoxychlor is read directly in the ultraviolet in cyclohexane solutions.[47] The method of double-impurity index is used to correct for background absorption. Beer's law is followed for solutions with transmittances of 20-60%.

Samples—*Fatty animal tissues.* Grind the sample and extract with several portions of diethyl ether until the fat is reduced to a fine powder, using a minimum of 50 ml. of diethyl ether for each gram of tissue. Evaporate the solvent from a portion of the extract equivalent to 4 grams of fat or 10 grams of other tissue.

Dissolve the residue in 45 ml. of petroleum ether and shake vigorously with 15 ml. of acetonitrile for 2-3 minutes. Let the layers separate and drain the lower acetonitrile layer. Extract the petroleum ether with three 15-ml. portions of acetonitrile. Shake the combined acetonitrile extracts with 20 ml. of petroleum ether to insure complete removal of waxes. Wash the petroleum-ether layer with two 5-ml. portions of acetonitrile and heat the combined acetonitrile washings and extracts at 100° with a gentle air current to concentrate the volume to 5 ml. Avoid overheating.

Reflux the acetonitrile concentrate with 50 ml. of 2% potassium hydroxide solution in 95% ethanol for 30 minutes to saponify the fats and dehydrohalogenate the methoxychlor. Dilute with 25 ml. of water and extract with 50 ml. of petroleum ether. Extract the petroleum ether layer with 50 ml. of 50% ethanol. Discard the ethanol and concentrate the petroleum ether at room temperature to 5 ml. with a gentle stream of air.

Assemble a chromatography tube 10 mm. in outside diameter × 130 mm. long with a glass-wool plug at the bottom. Add 5 grams of a 1:1 mixture of Celite and absorptive powdered magnesia and pack by tamping. Rinse the column with 5-10 ml. of petroleum ether and discard the rinsings. Pour the sample into the column and chromatograph the concentrate using air pressure. Rinse several times with petroleum ether, collecting 100 ml. of the eluate. Evaporate the petroleum ether to dryness with an air current, avoiding excess drying or heating. Develop with sulfuric acid.

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[46] Frieda M. Kunze, *J. Assoc. Offic. Agr. Chemists* **37**, 578-81 (1954).

[47] E. C. Jennings, Jr., and D. G. Edwards, *Anal. Chem.* **25**, 1179-82 (1953).

Nonfatty animal tissues. Macerate the sample with sand. Dehydrate with anhydrous sodium sulfate and grind until the mixture is dry and powdered. Extract several times with diethyl ether and grind thoroughly. Let stand and decant after each extraction, using a minimum of 20 ml. of diethyl ether for each gram of tissue. Follow the procedure for fatty animal tissues starting at "Evaporate the solvent from a portion of the extract. . . ."

Apples and pears.[48] Weigh 20-25 samples, add 500 ml. of benzene, and stopper. Rotate 5 minutes at 75-100 rpm. to remove insecticide from the surface without penetration of the solvent into the interior. Quickly drain off benzene and follow the procedures for fatty animal tissue starting with "Evaporate the solvent from a portion of the extract . . ." although in this case the solvent is benzene.

Plants. Extract a sample with petroleum ether or benzene. Shake the extract with one-half its weight of anhydrous sodium sulfate and filter. Remove the solvent from an aliquot containing 0.005-0.05 mg. of methoxychlor with a gentle air current at room temperature. Add 50 ml. of 2% potassium hydroxide solution in 95% ethanol and concentrate the solution to 5 ml. at 100°. Immediately take up in 200 ml. of water. Add 75 ml. of petroleum ether and 10 ml. of a saturated sodium chloride solution. Shake and discard the aqueous layer. Filter the petroleum ether through 1-2 cm. of anhydrous sodium sulfate. Wash the separatory funnel with 20 ml. of petroleum ether and pass through the filter. Remove the solvent from the filtrate with a current of air at room temperature or by reduced pressure. When dry, add 5 ml. of dry acetone and heat 2-4 seconds at 100° to dissolve. Chill to $-15°$ and chill dry acetone for washing. Filter with suction and wash the tube and filter with 3 ml. of cold acetone. Add the washings to the filtrate. Evaporate the filtrate with an air current, avoiding excess drying. Develop with sulfuric acid.

Insecticide-treated paperboard. Remove the treated liner from 50 square inches of paperboard and cut the liner into 0.5 × 2.5 inch pieces. Extract with 250 ml. of cyclohexane for at least 2 hours for samples estimated to contain 50-100 mg. of methoxychlor per square foot and for longer periods up to 6 hours for samples containing 200-1000 mg. per square foot. Concentrate the extract to 150 ml. by fractional distillation, cool to room temperature, and dilute to 200 ml. with cyclohexane. Read in the ultraviolet.

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[48] "Official Methods of Analysis of the Association of Official Agricultural Chemists," Association of Official Agricultural Chemists, 8th ed. pp. 406-7. Washington, D. C., (1955).

Procedure—*By sulfuric acid.* To prepare the reagent, add 10 mg. of ferric chloride to each liter of 6:1 sulfuric acid. To the residue containing 0.005-0.05 mg. of dehydrohalogenated methoxychlor, add 10 ml. of the sulfuric acid reagent. Stopper and shake well at intervals for 10 minutes. Let stand for 15 minutes–3 hours. Filter if necessary and read at 555 mμ against 6:1 sulfuric acid.

In the ultraviolet. Read a cyclohexane aliquot containing 0.04-0.14 gram per liter against cyclohexane at 215, 230, and 238 mμ. Calculate the impurity index at the wave length given as follows:

$$\text{I.I.}_{215\ \text{m}\mu} = \frac{(k_s)_{215}}{(k_s)_{230}} - \frac{(a_s)_{215}}{(a_s)_{230}}$$

$$\text{I.I.}_{238\ \text{m}\mu} = \frac{(k_s)_{238}}{(k_s)_{230}} - \frac{(a_s)_{238}}{(a_s)_{230}}$$

in which a_s = the absorbancy index and k_s = the absorbancy coefficient. To calculate the methoxychlor concentration:

$$c = (1 - \alpha)\frac{(k_s)_{230}}{(a_s)_{230}}$$

in which

$\alpha = 1.45\,(\text{I.I.})_{215} + 2.71\,(\text{I.I.})_{238}$.

The absorbancy index $= a_s = \dfrac{\log(1/T_s)}{bc}$

in which

T_s = the transmittancy of the sample

b = the cell thickness in cm.

c = the concentration in grams per liter.

1,4,5,6,7,7-HEPTACHLORO-3A,4,7,7A-TETRAHYDRO-4,7-METHANOINDENE, HEPTACHLOR

The ethanoldiamine method for heptachlor (Vol. III, p. 545) is modified to allow the determination of 0.0025-0.005 mg. heptachlor.[49] Interfering residues, pigments, oils, and waxes are removed by chromatography. If a large excess of oil or wax is present, the sample is prewashed with sulfuric acid.

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[49] E. P. Ordas, V. C. Smith, and C. F. Meyer, *J. Agr. Food Chem.* 4, 444-51 (1956).

Sample—*Food or forage crops.* Clean a 500-1000 gram sample by a method that corresponds to the accepted domestic or commercial preparation of the crop. Cut samples containing water, with the exception of canned goods, into 1-inch cubes and store in polyethylene bags for 24 hours at 18°F. to reduce the amount of dehydration later required. Reduce dry crops by grinding or cutting.

Grind the wet sample with twice its weight of anhydrous sodium sulfate. Store at 18°F for 24 hours to assure more complete removal of water. Extract for 4 hours with *n*-pentane which has been purified by washing with concentrated sulfuric acid followed by 5.6% potassium hydroxide solution saturated with potassium permanganate and distilled over potassium hydroxide pellets. Concentrate the solvent to 75 ml. in the extraction flask and thereafter to 5-10 ml. at 50°.

The presence of more wax than can be handled in the chromatographic column calls for a preliminary dewaxing. To determine whether this is necessary, chill the concentrated plant extract under water. If less than 0.1 ml. of sediment is found the step is not necessary. If a significant amount of wax is found, dilute the extract to 20 ml. and follow one of the procedures which follow for wax removal.

Dilute the concentrated extract to 300 ml. with pentane and shake vigorously with 40 ml. of a 1:1 mixture of 15% fuming sulfuric acid and concentrated sulfuric acid. Let stand for 16 hours, decant the pentane layer, and concentrate to 5 ml.

Wash samples which react violently with acid such as concentrated amine extracts from dried tobacco by the following procedure. Dilute the extract to 300 ml. with pentane and cool in a dry ice-isopropanol bath. Add 30 ml. of a 1:1 mixture of 15% fuming sulfuric acid and concentrated sulfuric acid dropwise over a period of 5 minutes with constant stirring. Remove from the bath and let stand until the temperature reaches room temperature. Let stand for 8 hours, discard the acid layer, and repeat the treatment of the pentane layer. Wash the pentane layer with 15% fuming sulfuric acid as above starting with "Dilute the concentrated extract to 300 ml. . . ."

In cases where the sample contains waxes which are not adequately removed by the sulfuric acid washing, reduce the plant extract to 20 ml. at 50° and cool in a dry ice-isopropanol bath until the wax is precipitated.

Filter while chilled and rinse with small amounts of cold pentane. Repeat if necessary.

Chromatography through Florisil for sample extracts containing little or no oil or wax. Activate 60-100 mesh Florisil by heating at 130° for 24 hours in an oven. Prepare a chromatograph column from a 50-ml. buret and insert it into a water jacket. Place a cotton plug at the bottom of the buret and add 10 grams of activated Florisil. Pack by tapping. Wet with purified *n*-pentane. Add the extract concentrated to 5 ml. Rinse the flask containing the extract with two or three 1-ml. portions of pentane and add the rinsings to the column. When the washings have penetrated the column, elute with 200 ml. of pentane and collect the total eluate.

Concentrate the eluate to 10 ml. at 50° from a standard-taper flask fitted with a Snyder three-bulb fractioning column to prevent loss of toxicant by codistillation or mechanical entrainment. Evaporate the remaining pentane to dryness at 40° and drive off any remaining pentane with an air current. Develop with ethanolamine.

Chromatography through Florex for all other heptachlor samples. Follow the procedure for Florisil chromatography using Florex XXS 60-90 mesh which has been activated by heating at 130° for 48 hours. Start with "Prepare a chromatography column . . ." and end with ". . . and add the rinsings to the column." Elute with 150 ml. of pentane, discard the first 50 ml. and collect the remaining 100 ml. Follow the procedure under Florisil chromatography starting at "Concentrate the eluate to 10 ml. . . ."

Procedure—*By monoethanolamine.* As a reagent, dissolve 33 grams of potassium hydroxide in 28 grams of water. Cool to room temperature and add an equal volume of butyl Cellosolve and 30.5 grams of monoethanolamine. Dilute to 1 liter with butyl Cellosolve. Let stand for several days or heat at 50° for 5 hours in a closed glass system. Decant and mix with an equal volume of benzene.

To the residue containing 0.0045-0.0225 mg. of heptachlor, add 0.2 ml. of reagent and heat at 100° for exactly 15 minutes. Cool to room temperature and dilute to 0.5 ml. with 4:1 benzene-isopropanol. Read at 567 mμ against benzene-isopropanol.

1,2,4,5,6,7,7-OCTACHLORO-3A,4,7,7A-TETRAHYDRO-4,7-METHANOINDAN, CHLORDAN

Chlordan is developed with diethanolamine and potassium hydroxide by a method similar to that for heptachlor. The procedure does not distinguish between chlordan and heptachlor.[50]

Interferences are removed by chromatography through Celite or alumina. To remove waxes the extract is dried in an ice bath under vacuum and dissolved in absolute methanol with heat. The solution is put in an ice bath to precipitate the waxes which are filtered out with suction. A slight to heavy cloudiness which appears after cleanup treatment indicates the presence of a large quantity of wax which is removed by using Pregl tubes of slightly more than 1-ml. capacity with asbestos overlays and forcing the colored solution through under pressure. An alternate procedure for removing wax involves a prewash with sulfuric acid. The addition of 1 ml. of 98% isopropanol increases the sensitivity by 27%.

Samples—*Food or forage crops.* Follow the procedure under heptachlor starting at the beginning and ending at the start of chromatography through Florisil. Concentrate the sample extract to 10 ml. and carry the concentrate through Florisil as described under heptachlor, ending with ". . . elute with 200 ml. of pentane and collect the total eluate."

Pack a column with 10 grams of alumina which has been activated by drying at 130° for 24 hours under the vacuum provided by a water aspirator. Concentrate the eluate from the Florisil treatment to 5-10 ml. Wet the alumina column with pentane, add the eluate to the column, and rinse the flask containing the eluate with two or three 1-ml. portions of pentane. Add the rinsings to the column. Elute with 50 ml. of pentane and discard the eluate. Elute with 50 ml. of diethyl ether and collect the eluate.

Follow the procedure under Florisil chromatography starting with "Concentrate the eluate to 10 ml. at 50° from a standard-paper flask. . . ." Develop with diethanolamine. If the sample has been pretreated with sulfuric acid to remove waxes, omit chromatography through Florisil and add a 10-ml. concentrated extract directly to alumina.

Fruits and vegetables. To purify pentane, shake 1 gallon of pentane with 200 ml. of 15% fuming sulfuric acid for 5 minutes. Let the layers separate for 1 hour and discard the lower layer. Shake the pentane with

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[50] David Williams, *J. Assoc. Offic. Agr. Chem.* **40,** 254-60 (1957).

200 ml. of concentrated sulfuric acid and discard the lower layer. Wash the pentane 2-3 times with a large volume of water. Treat the pentane similarly with 4% potassium permanganate solution and with 4% potassium permanganate solution in 5% sodium hydroxide solution. Wash with water after each treatment. Filter and distil over anhydrous potassium carbonate.

Place the sample in a refrigerator for several hours to firm. Weigh enough sample into a dry jar to prevent undue breakage of the food during tumbling. Stopper and extract for 5 minutes with pentane at approximately 250-300 ml. for a 1-gallon jar. Filter the extract. Place an amount of extract equivalent to 200 grams of original sample in an ice bath and evaporate the pentane under high vacuum using a water aspirator and all glass connections. Add 3 ml. of absolute methanol, stopper, and heat carefully with swirling until waxes are dissolved. Chill the solution in an ice bath. Filter the methanol through a fritted glass filter with an asbestos overlay with slight suction. Wash the flask and filter with no more than 25 ml. of ice-cold methanol, sucking the cake dry after each washing.

Take up the filtrate in 100 ml. of purified pentane and mix by swirling. Add 75 ml. of water and shake vigorously 100 times. When the layers begin to separate, draw off the bottom water-methanol layer and extract this layer with 50 ml. of pentane. When the separation is sharp discard the bottom layer and add the top pentane layer to the original pentane phase. Wash the second separatory funnel with 40 ml. of water and add the washings to the pentane extracts.

Shake the pentane extracts with several quick vertical shakes to break any emulsion. Draw off the bottom water layer and discard. Wash the pentane with three 30-ml. portions of water, shaking 10-15 times. Remove as much water as possible. Add an excess of anhydrous sodium sulfate to the pentane to remove moisture until the sodium sulfate appears granular rather than wet. Rinse the separatory funnel with a small amount of pentane and add the rinsings to the pentane extract.

To prepare the chromatography column, mix 15 grams of Celite 545 with 4.5 ml. of concentrated sulfuric acid and 4.5 ml. of 15% fuming sulfuric acid. Add enough pentane to form a paste. Pour 25 ml. of pentane into a 20 × 230 mm. glass tube with a constricted end plugged with glass wool. Add the paste in 2 portions, packing gently after each addition. Control the solvent flow so that some of the pentane stands at the top of the column and the flow from the column is approximately 120-150 drops per minute.

Concentrate the extract under vacuum in an ice bath to 2-3 ml. Transfer the concentrate to the chromatograph column. Wash the flask containing the concentrate at least 3 times with pentane, adding the washings to the column and allowing each wash to penetrate the column before adding the next. Add 100 ml. of pentane in several portions to elute chlordan.

Evaporate the eluate in an ice bath under vacuum to 2-3 ml. and wash into a tube with 3 sprays of pentane. Dilute to 10 ml. with pentane and develop with diethanolamine and potassium hydroxide.

Procedure—*By diethanolamine.* As a reagent, combine 2 parts of 5.6% potassium hydroxide solution in methanol with 1 part of diethanolamine and dilute with 9 parts of methanol. Heat a residue containing 0.005-0.05 mg. of chlordan with 0.2 ml. of reagent for 15 minutes at 100°. Cool to room temperature under water and dilute to 0.5 ml. with 90% methanol. Read at 550 mμ against 90% methanol.

Alternatively, as a color reagent, mix 1 part of vacuum-distilled diethanolamine with 2 parts of 5.6% potassium hydroxide solution in methanol. Let stand at 30-35° for 1 week. Add 9 parts of absolute methanol and mix.

Evaporate the pentane from a 10-ml. sample containing up to 0.16 mg. of chlordan in a stoppered tube until the pentane no longer condenses at the tapered part of the tube. Add 1 ml. of isopropanol and mix by swirling. Add 0.5 ml. of reagent and mix. Heat in a constant temperature bath at 100° at a depth of 40 mm. for 30 minutes. Stopper and cool to room temperature in cold water. Add 1 ml. of absolute methanol, stopper, and let stand for 20 minutes, shaking frequently to dissolve. Filter through 1.5 ml. Pregl tubes having an asbestos overlay with gentle pressure to remove waxes. Read at 550 mμ against anhydrous methanol.

O-(3-CHLORO-4-NITROPHENYL) *O,O*-DIMETHYL PHOSPHOROTHIOATE, CHLORTHION

Chlorthion is reduced to the amine by zinc and phosphoric acid. Thereafter it is developed by diazotation and coupling with *N*-1-naphthylethylenediamine.[51] During diazotation and coupling the ethanol concentration must not exceed 30% as side reactions may produce interfering colors. If final ethanol concentrations greater than 50% by volume are necessary to obtain clarity, more concentrated hydrochloric acid should be added to obtain the maximum absorption. Similar reactions are exhibited by *O*-(2-chlor-4-nitrophenyl *O*,*O*-dimethyl phosphorothioate and *O*,*O*-dimethyl *O*-*p*-nitrophenyl phosphorothioate, methyl parathion, with absorption maxima at 560 mμ. All traces of carbon tetrachloride must be removed.

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[51] M. J. Kolbezen and J. H. Barkley, *J. Agr. Food Chem.* **2**, 1278-80 (1954); M. J. Kolbezen and H. T. Reynolds, *Ibid.* **4**, 522-5 (1956).

In milk samples, fats are separated by equilibration with hexane from an acetonitrile-isopropanol extract of the sample. The solution is acidified and the amine is extracted into the aqueous phase. Isopropanol aids in preventing emulsions. Concentrations greater than 0.1 ppm. in 500 ml. of milk can be determined spectrophotometrically and concentrations from 0.02-0.1 ppm. can be estimated by visual comparison. Cottonseed are delinted with sulfuric acid; then extracted with pentane. Chlorthion is separated from cottonseed oil by partition between pentane and acetonitrile. Pigments and other interferences are removed from an acetonitrile solution of chlorthion by chromatography through activated alumina. Beer's law is followed at 545 mμ.

Procedure—*Milk.* Homogenize a 500-ml. sample in two portions each with 2.5 grams of sodium chloride in a blender. Add 250 ml. of 95% ethanol to each portion and blend for 1 minute. Extract the combined sample with 350 ml. of hexane for 6 hours at a reflux rate of 1 liter per hour. If, after cooling, the extract contains some solids, filter through a 1-inch column of anhydrous sodium sulfate. Reduce the volume to 25 ml. and add 50 ml. of isopropanol. Evaporate until all the hexane is driven off.

Alternatively, blend a 500-ml. sample in two portions, each with 500 ml. of carbon tetrachloride for 1 minute. Let the combined sample stand for 30 minutes. Filter the carbon tetrachloride through a 2-inch column of anyhdrous sodium sulfate. Concentrate the solvent to 20 ml., add 50 ml. of methanol, and evaporate to 20 ml. to remove traces of carbon tetrachloride. Add 30 ml. of isopropanol to the residue.

To the extract in isopropanol, obtained by either method, add 1 ml. of 85% phosphoric acid and 0.5 gram of powdered zinc. Reflux briskly for 30 minutes to reduce the nitro group to the amine.

Filter off the zinc with suction and rinse the reaction flask in several portions with 250 ml. of hexane and alternately with portions of 150 ml. of a 3:1 water-acetonitrile solution containing 2 ml. of 2:3 hydrochloric acid. Filter the washings into the amine solution. Shake the remaining hexane and water-acetonitrile solvents with the amine for 30 seconds. Equilibrate the lower phase with 100 ml. of hexane. Repeat the extraction with hexane and water-acetonitrile and again equilibrate the lower phase with 100 ml. of hexane.

Extract the hexane phases with two 100-ml. portions of a 1:1:2 mixture of isopropanol, acetonitrile, and water which has 1% of concentrated hydrochloric acid. Combine the lower phases and discard the hexane extracts.

Neutralize the acid by adding 200 ml. of 4% sodium bicarbonate solution. Shake cautiously and then vigorously until the evolution of carbon dioxide stops. Extract the aqueous phase containing precipitated zinc carbonate with two 150-ml. portions of hexane. Wash the hexane extracts with 100 ml. of 2% sodium bicarbonate solution followed with 75 ml. of a saturated sodium chloride solution. Shake with 1-2 grams of anhydrous sodium sulfate and filter.

Wash the funnel with 50 ml. of hexane and add 50 ml. of 50% ethanol in 1:59 hydrochloric acid. Evaporate the hexane completely at 100°, adding finely divided silicon carbide. Complete the hexane evaporation with a gentle air current. Cool, filter, and rinse the flask with three 15-ml. portions of 1:110 hydrochloric acid.

To a 60-ml. aliquot containing 0.03-0.3 mg. of reduced chlorthion, add 1 ml. of 0.25% sodium nitrite solution. After 10 minutes, add 1 ml. of 2.5% ammonium sulfamate solution. After 10 minutes, add 2 ml. of 1% *N*-1-naphthylethylenediamine dihydrochloride solution. After 10 minutes add 6 ml. of concentrated hydrochloric acid and dilute to 100 ml. with water. Read immediately at 545 mμ.

Cottonseed. To delint the cottonseed, stir mechanically ginned cottonseed with concentrated sulfuric acid for approximately 5 minutes and pour the mass into water. Pour onto a large wire screen and immediately wash with large quantities of water. Dry overnight at 40°. Grind the sample to a fine granular form.

Assemble an extraction apparatus as shown in Figure 26. Attach a flask containing 350 ml. of pentane to the extractor. Cover the porous plate of the extractor with a wad of fine glass wool and then with a 1-inch layer of coarse anhydrous sodium sulfate. Insert a length of 10-mm. glass tubing extending above the top of the extractor and add 200 grams of ground sample. Add pentane through the glass tube so that the extractor becomes filled from the bottom until overflow at the side arm begins. Remove the glass tube and rinse with pentane. Reflux for 6 hours to extract. Cool, filter if necessary, and concentrate to half the volume of the flask at 100°.

To separate chlorthion from extracted cottonseed oil, dilute the pentane extract to 250 ml. Equilibrate with 60 ml. of acetonitrile. If the acetonitrile is basic, it must be acidified with 85% phosphoric acid until neutral or slightly acidic. Add the lower phase to 100 ml. of pentane, equilibrate, and remove the lower phase. Repeat the extraction of both remaining phases with three 30-ml. portions of acetonitrile. Both the combined acetonitrile extracts containing chlorthion at 100° for several minutes to evaporate any pentane present. Cool.

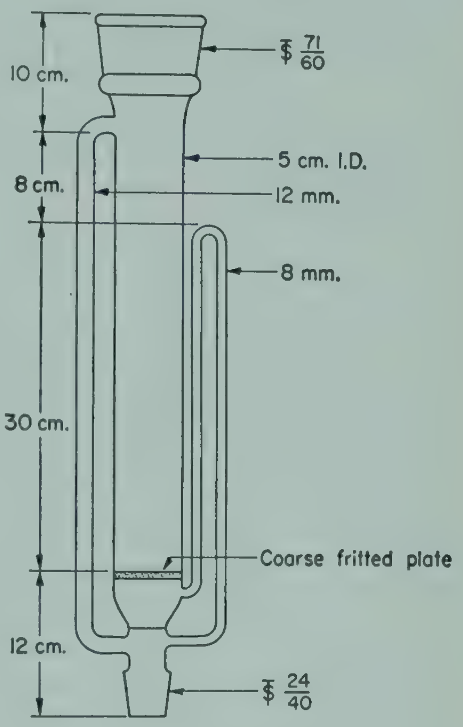

Fig. 26

Apparatus for continuous extraction of chlorthion from cottonseed

To activate, suspend 500 grams of alumina in 800 ml. of water and acidify with 5 ml. of concentrated hydrochloric acid. Let stand for 2 hours, shaking vigorously occasionally. Wash 5 times by decantation, allowing the fine materials to be poured off. Wash until the filtrate is only faintly acid. Wash three times with 300 ml. of methanol and dry at 180° overnight.

Prepare a column 18 mm. in inside diameter and 330 mm. long with a base of coarse fritted glass. Suspend −80 mest activated alumina in 50% methanol and wet pack into the column by vibration and gentle tamping to a depth of 2 inches. Pass 30 ml. of acetonitrile through the column. When this reaches the top of the alumina, add the acetonitrile extract. Rinse the flask containing the extract with two 10-ml. portions of acetonitrile. Collect the eluate in 500 ml. of water and 10 ml. of concentrated hydrochloric acid. When the acetonitrile reaches the top of the alumina, add 30 ml. of acetonitrile. Repeat with 30 ml. of acetonitrile. Extract the eluate with 100 ml. of pentane. Extract the lower phase with 100 ml. of pentane. Combine the pentane extracts, rinse the separatory funnels with 25 ml. of pentane, and add to the extract. Evaporate at 100° through a three-bulb Snyder column to 10 ml. Add 10 ml. of acetonitrile and evaporate to 10 ml.

Remove the Snyder column and rinse the bulb of the evaporator with 15 ml. of pentane. Add 5 ml. of water, 1 ml. of 85% phosphoric acid, and 0.5 gram of powdered zinc to the concentrate. Attach an air condenser to reduce losses by volatilization and heat at 100° for 30 minutes to reduce the nitro group to the amine. Add 20 ml. of water through the condenser and replace with a 5-inch joint. Boil for a few minutes to expel the last traces of pentane and cool.

To prepare the filter cotton which removes some oil, dry 10 grams of cotton at 150° for 2 hours. Immerse in 25 ml. of a pentane solution of 3 grams of white petroleum jelly and 3 grams of paraffin. Rotate in the air until the solvent is nearly evaporated. Evaporate in the air for 1 hour and at 150° for 30 minutes.

Filter the sample by suction through glass wool, a ½ to ¾ inch layer of packed Celite, and 1 inch of the cotton coated with petroleum jelly-paraffin. Rinse the reaction flask with three 4-ml. portions of 1% phosphoric acid solution and add the washings through the filter.

To the acid solution containing 0.005-0.04 mg. of chlorthion as the amine, add 0.5 ml. of 0.25% sodium nitrite solution. After 10 minutes, add 0.5 ml. of 2.5% ammonium sulfamate solution and after 10 minutes, add 1 ml. of 1% *N*-1-naphthylethylenediamine dihydrochloride solution. After 10 minutes, add 3 ml. of concentrated hydrochloric acid and dilute to 50 ml. with water. Read at 545 mμ.

CHLOROQUINE

Chloroquine exhibits three absorption maxima in the ultraviolet at 257, 330, and 342 mμ. After extraction from the sample with hydrochloric acid and chloroform, chloroquine is read at 342 mμ since the optical density is greatest at this wave length and the interferences are the least.[52] Beer's law is followed for 0.001-0.02 mg.

A reagent for the turbimetric determination of chloroquine contains potassium iodide and mercuric chloride.[53] Chloroquine is read fluorimetrically at 400 mμ at pH 11 by activation at 335 mμ.[54]

Samples—*Tissue.* Homogenize a 50-gram sample with 100 ml. of 1:110 hydrochloric acid. Add 30 ml. of 40% trichloroacetic acid solution and stir. Add 20 ml. of concentrated hydrochloric acid. Let stand overnight to precipitate proteins. Autoclave under 15-lb. pressure for 30 minutes to liberate chloroquine bound to proteins and nucleic acids. To remove fats, shake with 100 ml. of chloroform, and centrifuge. Make the aqueous phase alkaline with saturated potassium hydroxide solution and shake with two 100-ml. portions of chloroform. Wash the combined chloroform extracts with 50 ml. of 0.4% sodium hydroxide solution and shake the chloroform extracts with 25 ml. of 1:10 hydrochloric acid. Read in the ultraviolet.

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[52] R. W. Prouty and K. Kuroda, *J. Lab. Clin. Med.* 52, 477-80 (1958).

[53] J. P. Perira and E. Paulini, *Rev. brasil. malariol. e doencas trop.* 8, 613-5 (1956).

[54] S. Udenfriend, D. E. Duggan, B. M. Vasta, and B. B. Brodie, *J. Pharmacol. Exptl. Therap.* 120, 26-32 (1957).

Antimalarial drugs.[55] Dissolve a tablet in 1 liter of water, filter, and dilute 5 ml. to 100 ml. Read in the ultraviolet.

Urine. Shake a 5-ml. sample with 5 ml. of 2% hydroxide solution and 25 ml. of petroleum ether for 30 minutes. Add a few drops of 95% ethanol to break the emulsion and centrifuge for 2 minutes. Wash the petroleum ether layer twice with 3 ml. of 2% sodium hydroxide solution. Shake for 15 minutes with 10 ml. of 1:110 hydrochloric acid. Develop with potassium iodide and mercuric chloride.

Procedure—*In the ultraviolet.* Read a chloroform solution containing 0.001-0.02 mg. of chloroform at 342 mμ.

By potassium iodide and mercuric chloride. To prepare the reagent, dissolve 6.64 grams of potassium iodide in 90 ml. of water. Add 2.7 grams of mercuric chloride, stir well, and add 40 ml. of glacial acetic acid. Dilute to 140 ml. with water. To a 9-ml. acidic sample, add 1 ml. of 1% carboxymethylcellulose solution and 0.2 ml. of reagent. Shake for 10 seconds and after 50-60 minutes read the turbidity aganst a blank at 420 mμ.

ETHYL *p,p′*-DICHLOROBENZILATE [2-HYDROXY-2,2-BIS(4-CHLOROPHENYL) ETHYL ACETATE], CHLOROBENZILATE

Chlorobenzilate is hydrolyzed to *p,p′*-dichlorobenzilic acid which is oxidized by chromic anhydride in glacial acetic acid to *p-p′*-dichlorobenzophenone. This can be read directly at 264 mμ, or as its 2,4-dinitrophenylhydrazone at 510 mμ.[56] The hydrolysis step eliminates interferences not soluble in alkali. The oxidation provides further cleanup since only the water-insoluble ketones remain. The oxidizing agent must be in excess. Acetone interferes. DDT-type compounds which are converted in part to diphenylacetic acid derivatives by hydrolysis interfere. Beer's law is followed at 264 mμ for 0.001-0.14 mg. and at 510 mμ for 0.015-0.2 mg.

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[55] Ernest Paulini, *Rev. brasil. malariol. e doencas trop.* 7, 329-32 (1955).

[56] R. C. Blinn, F. A. Gunther, and M. J. Kolbezen, *J. Agr. Food Chem.* 2, 1080-3 (1954).

The nitration product of chlorobenzilate produces a red color with sodium methylate which shows maximum absorption at 418 and 538 mμ.[57] In the presence of DDT, chlorobenzilate is saponified to 4,4′-dichlorobenzilic acid and remains in the aqueous phase as the potassium salt. It is then acidified and extracted from DDT with ethyl ether. The acid gives the same color upon nitration and reaction with sodium methylate as the ester. Stearic acid is added to minimize possible losses of chlorobenzilate due to volatilization during evaporation of the solvents or due to "burning up" during nitration. Beer's law is followed for 0.1-0.5 mg. The method is applicable for the determination of chlorobenzilate in apples, pears, peaches, cantaloupes, and strawberries.

Procedure—*As* p,p′-*dichlorobenzophenone.* To an aliquot of chlorobenzilate in petroleum ether solution such as those obtained by citrus fruit extraction, add 5 ml. of 10% potassium hydroxide solution in 95% ethanol and evaporate to 5 ml. at 100° through a three-ball Snyder column. Remove the column and evaporate at 100° until the residue is gel-like. Add 2 ml. of water and 50 ml. of hexane. Swirl gently and remove most of the hexane. Repeat the extraction with two 50-ml. portions of hexane and remove the remaining hexane from the aqueous extract by warming at 100°.

Add 25 ml. of glacial acetic acid and 3 grams of chromic anhydride to the residue and reflux for 10 minutes. If the solution is green, add 2 grams more of chromic anhydride and reflux for 10 minutes. Cool to room temperature. Shake vigorously with 100 ml. of water and 75 ml. of hexane. Shake the lower aqueous layer with 25 ml. of hexane for 30 seconds. Discard the aqueous phase and wash the first hexane extract with three 75-ml. portions of water. Wash the second hexane extract with the same water. Filter the first hexane extract through 1 gram of anhydrous sodium sulfate. Rinse the first separatory funnel with the second extract and filter the second extract into the first extract. Rinse both separatory funnels with a 50-ml. portion of hexane and filter into the combined extracts. Evaporate the solvent and remove the last traces with a gentle air current.

Gently boil the residue with 4 ml. of 95% ethanol until the volume reaches 1-2 ml. This will remove traces of volatile aromatic substances. Dilute to 10 ml. with 95% ethanol and read the *p,p′*-dichlorobenzophenone at 264 mμ.

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[57] H. J. Harris, *Ibid.* **3**, 939-41 (1955).

As the 2,4-dinitrophenylhydrazone. Follow the procedure "as *p,p'*-dichlorobenzophenone," ending with "Evaporate the solvent and remove the last traces with a gentle air current."

To prepare the 2,4-dinitrophenylhydrazine reagent, pass dry hydrogen chloride gas into aldehyde- and ketone-free methanol until 9-11 grams of gas per 100 ml. has been absorbed. Dissolve 10 mg. of 2,4-dinitrophenylhydrazine per ml. of the methanol-hydrogen chloride solution.

Add 1.5 ml. of methanol and 1 ml. of 2,4-dinitrophenylhydrazine reagent to the sample residue and heat in a stirred oil bath at 65° for 5 minutes. Stopper and let stand in the oil bath for 3 hours. Add 4 ml. of 1,2-dichloroethane, mix gently, and add 10 ml. of 4% sodium hydroxide solution. Shake vigorously for 30 seconds. Remove the upper aqueous phase, add 5 ml. of water, and mix thoroughly. Remove the upper aqueous phase as completely as possible. To 2 ml. of the 1,2-dichloroethane solution add 1 ml. of 4% potassium hydroxide solution in 95% ethanol. Dilute to 25 ml. with 95% ethanol and read at 510 mμ.

By sodium methylate. Concentrate an aliquot of benzene solution obtained from extraction of fruits to 10 ml. at 100°. Add 2 ml. of 0.5% stearic acid solution in benzene and concentrate to 5 ml. Remove the remaining benzene at 50° with the aid of a gentle air current. Add a few ml. of absolute methanol and remove the solvent at 50° with an air current.

Place in an ice bath and add 5 ml. of a cold 1:1 mixture of fuming nitric acid and concentrated sulfuric acid, taking care to wet the sides of the tube. Heat to 85° in 30-35 minutes and heat at 100° for 1 hour with intermittent swirling.

Chill in cold water and add 25 ml. of ice-cold water to stop the nitration. Swirl gently and rinse quantitatively with 25 ml. of water and 75 ml. of ether into a separatory funnel. Shake vigorously for 1 minute, allow the layers to separate completely, and discard the lower layer. Add 10 ml. of 5% potassium hydroxide solution and shake vigorously for 30 seconds. Discard the lower layer and repeat the extraction with two 10-ml. portions of 5% potassium hydroxide solution.

Wash the ether layer with three 15-ml. portions of a saturated sodium chloride solution and draw off the final salt wash as completely as possible. Filter the ether phase slowly through glass wool. Rinse the separatory funnel with one 15-ml. portion and two 10-ml. portions of ether, adding the washings to the filtrate. Evaporate the ether at 100°, removing the last traces with a gentle stream of air while the flask is being heated. Cool, add 25 ml. of dry benzene, and swirl to dissolve the residue.

When the chlorobenzilate concentration is low, reduce the amount of dry benzene to increase the intensity of the color produced with methylate.

To a 5-ml. aliquot containing 0.1-0.5 mg. of chlorbenzilate, add 10 ml. of 10% sodium methylate solution and swirl gently to obtain a homogeneous solution. Read after 15 minutes at 538 mμ.

By sodium methylate in the presence of DDT. Reduce a benzene solution containing 0.1-0.5 mg. of chlorobenzilate to 10 ml. at 100° and evaporate the remaining solvent with the aid of a gentle air current. Reflux the residue with 50 ml. of 2% potassium hydroxide solution in methanol for 30 minutes. Cool and transfer quantitatively with 50 ml. of water to a separatory funnel. Add 100 ml. of petroleum ether and shake vigorously for 1 minute. Draw off the lower layer and shake it with 50 ml. of petroleum ether for 30 seconds. Draw off the lower water-alcohol layer. To the first separatory funnel add 10 ml. of water. Shake gently for 5 seconds and swirl to break any emulsion formed.

Transfer the water wash to the second separatory funnel, shake, and add the water wash to the water-alcohol layer previously removed. Repeat the wash procedure with two 10-ml. portions of water. Remove the alcohol at 100° and reduce the volume to 60 ml. Cool and take up in 25 ml. of water. Acidify to litmus with 1:3 hydrochloric acid. Add 100 ml. of ethyl ether and shake vigorously for 1 minute. Shake the aqueous layer with two 50-ml. portions of ether and discard the aqueous layer.

Filter the first ether extract through glass wool and a 1.5-inch layer of anhydrous sodium sulfate. Rinse the first separatory funnel with the second ether extract and filter into the first extract. Rinse the second and first separatory funnels with the third ether extract and filter into the combined extracts. Concentrate the ether extracts to 5 ml. at 100° and take up in 25-30 ml. of acetone. Evaporate the acetone to 10 ml. at 100° and add 2 ml. of 0.5% stearic acid solution in acetone. Evaporate until 5 ml. remains and remove the remaining acetone at room temperature with the aid of a gentle air current. Develop with sodium methylate starting at "Place in an ice bath and. . . ."

NITROGEN MUSTARD, 2,2′-DICHLORO-*N*-METHYL DIETHYLAMINE

8-Quinolinol reacts with bifunctional β-halogen ethyl compounds to produce a color which varies from bright orange for high concentrations of nitrogen mustard to a yellow-green for lower concentrations with an absorbance maximum at 468 mμ. When quantitatively extracted into chloroform, the color is bright pink, with a maximum absorbance at

285 mμ.[58] The bis-(β-halogenethyl) configuration is essential for color formation as the monofunctional or the bis-(β-hydroxyethyl) analogs do not yield color with the reagent. The lengthening of the substituent *N*-alkyl side chain from *N*-hexyl to *N*-hexadecyl causes loss in color-producing activity.

Maxima obtained from other compounds on reaction with 8-quinolinol are methylbis (β-chloroethyl)amine hydrochloride 468 mμ, *n*-propylbis (β-chloroethyl)amine 485 mμ, *n*-butylbis(β-chloroethyl)amine 470 mμ, *n*-amylbis(β-chloroethyl)amine 478 mμ, γ-chloro-*n*-propylbis-(β-chloroethyl)amine 477 mμ, *N*,*N*,*N*′,*N*′-tetrabis(β-chloroethyl)amine 477 mμ, methylbis(β-bromoethyl)amine hydrobromide 480 mμ, bis(β-chloroethyl)sulfide 470 mμ, and bis(β-chloroethyl) sulfoxide at 470 mμ. The concentration of the 8-quinolinol reagent is critical. The colored compound is unstable at 100°. A high acidity inhibits color development. Some reducing agents such as zinc dust inhibit color formation. Oxidizing compounds, such as hydrogen peroxide and potassium periodate also form an orange color with the reagent, but the color cannot be extracted into chloroform. Cysteine inhibits color formation since it binds nitrogen mustard. Beer's law is followed at 468 mμ.

Procedure—*By 8-quinolinol.* To a 2.5-ml. sample containing not more than 0.2 micro mole of nitrogen mustard per ml., add 2.5 ml. of 95% ethanol and a few drops of 5% acetic acid solution. Add 0.5 ml. of 5% 8-quinolinol solution in ethanol and agitate. Shake vigorously with 0.5 ml. of 10% sodium carbonate solution for 30 seconds, let stand for 90 minutes at room temperature, and read at 468 mμ.

SESQUIMUSTARD, BIS(2-CHOROETHYLMERCAPTO)-ETHANE

An *o*-dianisidine-copper acetate reagent produces a strong red color with sesquimustard in ethanol after addition of strong acid.[59] Beer's law is followed. Mustard gas gives a minor degree of color under these conditions.

Procedure—To a 1-ml. sample containing 0.02-0.1 mg. of sesquimustard per ml. add 1 ml. of 0.1% *o*-dianisidine solution in absolute ethanol. After 30 seconds, add 3 ml. of 0.1% copper acetate solution containing 0.001 ml. of glacial acetic acid per ml. of solution. Stir well and after 10 minutes add 3 ml. of 1:1 sulfuric acid. Stir and read against a reagent blank at 540 mμ.

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[58] Eberhard G. Trams, *Anal. Chem.* **30**, 256-9 (1958).

[59] Abraham Koblin, *Ibid.* **30**, 430-2 (1958).

BROMINATED HYDROCARBONS

Brominated hydrocarbons such as ethylene dibromide and methyl bromide, are oxidized and absorbed in an alkaline solution of hydrogen peroxide. The inorganic bromide reacts with sodium gold chloride to form auribromic acid.[60] The method is applicable to 0.1-4 mg. of inorganic bromide. Large amounts of chlorides cause a decrease in the color intensity. Iodides, which form a deep greenish-yellow color with the reagent, mask the bromide color.

Procedure—*Air.* Draw the sample through a catalytic furnace and absorb the gas in 100-volume hydrogen peroxide in alkaline solution. Cover and boil for 5-10 minutes, reducing the volume to 2-3 ml. to prevent precipitation of the reagent by hydrogen peroxide as colloidal gold. Rinse the cover and dilute the solution to a known volume. Acidify an aliquot containing 0.1-4 mg. of bromide with 1:5 sulfuric acid and dilute to 8 ml. Add 0.6 ml. of 1% sodium gold chloride solution and read against a reagent blank at 470 mμ.

METHYL BROMIDE

Methyl bromide reacts with pyridine to form methylpyridine bromide. This forms a colored complex when treated with sodium hydroxide in aqueous solution.[61] An excess of water adversely affects the intensity of the color but approximately 1% of water is necessary to prevent precipitation of the methylpyridine bromide. The solution is stabilized by addition of gum arabic. The method is applicable for measuring up to 1.5 mg. of methyl bromide per ml. and to concentrations as low as 50 mg. per cubic meter of air.

Methyl iodide, ethyl iodide, and ethyl bromide give colors of similar intensity to that of the methyl bromide, while ethyl chloride, ethylene chloride, and ethylene dibromide give colors of lesser intensity. Higher aliphatic halides interfere slightly or not at all. Carbon tetrachloride, chloroform, trichoroethylene, and tetrachloroethane give a red color with absorption maxima at 365 and 535 mμ.

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[60] Peter Wade, *J. Sci. Food Agr.* 3, 390-3 (1952).

[61] G. A. Lugg, *Analyst* 80, 290-5 (1955).

Procedure—*Air.* Draw a 10-liter sample into a gas chamber. Add 15 ml. of pyridine solution containing 1 ml. of water per 100 ml. of pyridine. Stopper immediately and wet the sides of the chamber with the pyridine. Let stand 7 hours for the formation of methylpyridine bromide. Invert the bottle, shake, and allow it to drain. To a 10-ml. portion, add 0.5 ml. of 2% sodium hydroxide solution and heat for 15 minutes at 95°. Cool for 5 minutes in a water bath at room temperature and add 0.5 ml. of 2% gum arabic solution. Read after 10 minutes at 420 mμ against water.

THYROXINE

The orange color of thyroxine in acid solution on addition of sodium nitrite (Vol. III, p. 533) can be developed as a red color with potassium hydroxide.[62]

Procedure—To a 1-ml. solution containing 0.001-0.01 mg. of thyroxine, add 1 ml. of dilute hydrochloric acid and 0.1 ml. of 32% sodium nitrite solution. After 5 minutes, add 0.1 ml. of 22.4% potassium hydroxide and read at 500 mμ.

3,5-DIIODOTYROSINE

When the iodo group of 3,5-diiodotyrosine is replaced by a nitroso group the resulting compound forms a stable green chelate with cobalt.[63] The color is most stable in a mixture of sodium acetate and acetic acid. Diiodotyrosine is separated from thyroxine by butanol fractionation, which also removes interference from 3,3′,5-triiodotyronine and 3,5-diiodotyronine. The presence of over 25 mg. of casein hydrolysate produces a slight increase in the exported amount of 3,5-diiodotyrosine. Tyrosine does not interfere. The absorption increases in the presence of sodium chloride. Therefore the amount in the sample must correspond to that present in preparation of the standard curve. Beer's law is followed for 0.1-0.9 mg.

Procedure—*Natural and artificial iodoproteins.* Hydrolyze a 200-500 mg. sample with 4-10 ml. of 20% sodium hydroxide solution at 115-120° for 8 hours. Add 3-6 ml. of 40% sodium hydroxide solution so that, when diluted with 20 ml. of water, the final concentration will contain 16% of sodium hydroxide. Add 20 ml. of *n*-butanol and shake every 15 minutes for 2 hours. Centrifuge and draw off the butanol layer. To the alkali layer add 8 ml. of concentrated hydrochloric acid. Stir well and filter. Dilute the filtrate to 100 ml. This solution will contain 5.8% of sodium chloride.

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[62] H. Spitzy and H. Stormann, *Microchemie ver. Mikrochim. Acta* **40**, 198-201 (1953).

[63] N. R. Moudgal, L. K. Ramachandran, and P. S. Sarma, *Analyst* **79**, 43-6 (1954).

Dilute an aliquot containing 0.1-0.9 mg. of 3,5-diiodotyrosine to 5 ml. Add 0.2 ml. of 1:11 hydrochloric acid and 0.2 ml. of 1% sodium nitrite solution. Incubate at 38° for 30 minutes. Add 0.05 ml. of 1% cobalt chloride solution and 5 ml. of a mixture of 3 volumes of saturated sodium acetate solution and two volumes of 20% acetic acid. Read at 390-395 mμ against a reagent blank.

3,5-DIIODOTHYRONINE

A mercury reagent produces a red color with 3,5-diiodothyronine.[64] Addition of surface-active agents such as sodium lauryl sulfate and trimethylcetylammonium bromide prevented precipitation. 3,5-Diiodothyronine is separated from thyroxine by partitioning between butanol and aqueous sodium hydroxide.

Procedure—*Thyroxine absent.* To prepare the mercury reagent, dissolve with warming 37.5 grams of mercuric sulfate, 27.5 grams of mercuric chloride, and 35 grams of anhydrous sodium sulfate in 700 ml. of water containing 53 ml. of sulfuric acid. Cool and dilute to 1 liter with water. To prepare the sodium lauryl sulfate diluent, dissolve 0.5 gram of sodium lauryl sulfate in 400 ml. of water. Mix 19 ml. of sulfuric acid with 400 ml. of water. Mix the two solutions and dilute to 1 liter with water.

To 1 ml. of 0.4% sodium hydroxide solution containing up to 1 mg. of diiodothyronine, add 15 ml. of mercury reagent and boil gently for 60 ± 5 seconds. Immediately add 0.1 ml. of 6.9% sodium nitrite solution. Mix and dilute immediately with 25 ml. of the sodium lauryl sulfate diluent. Cool in water for 5 minutes, dilute to 50 ml., and read at 480 mμ.

Thyroxine present. Shake *n*-butanol with 4% sodium hydroxide solution. Separate and filter the butanol. Extract a 5-ml. sample in 4% sodium hydroxide solution containing up to 50 mg. of thyroxine with three 5-ml. portions of the prepared *n*-butanol. Follow the procedure for thyroxine absent starting with "To 1 ml. of 0.4% sodium hydroxide solution. . . ." Multiply the result by 2. Theoretically 51% of the diiodothyronine remains unextracted but some additional loss occurs.

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[64] W. H. C. Shaw, *Analyst* **78,** 253-4 (1953).

5-Iodosalicylic Acid

5-Iodosalicylic acid is extracted with ethanol and diethyl ether and read directly at 345 and 313 mμ.[65] 4-Iodosalicylic acid has an absorption maximum at 302 mμ. 5-Iodosalicylic acid can also be extracted from acidified urine with carbon tetrachloride and read at 330 mμ.[66]

Procedure—*Serum.* To a 0.5-ml. sample containing 5-iodosalicylic acid, add 9.5 ml. of a 3:1 mixture of 95% ethanol and diethyl ether. Let stand for 5 minutes and heat to 100°. Cool, filter, and read at 345 and 313 mμ. The extinction must be corrected for nonspecific absorption by normal serum. Therefore the value is $E = (0.47 \times [E_{313} - E_{345}]/0.36$.

Alternatively read at 286, 304, and 332 mμ. Then $E = E_{304} - 0.609 (E_{286} - E_{332}) - E_{332}$.

DD-Threo-2-dichloroacetamido-1-(4-methylsulfonylphenyl)-1,3-propanediol, Dextrosulphendiol, and Raceophenidol

Alkaline hydrolysis of dextrosulphendiol followed by periodate oxidation gives *p*-methylsulfonylbenzaldehyde. This is read in the quinoid form at 570 mμ.[67] With slight modifications this is also applicable to raceophenidol.

Procedure—*Urine. Total dextrosulfendiol.* Mix a 1-ml. sample with 2 ml. of 4% sodium hydroxide solution. Hydrolyze by heating at 100° for 1 hour. Cool and dilute to about 10 ml. Add 3 ml. of 20% solution of sodium dihydrogen phosphate monohydrate. Extract with 80 ml. of ether, then with 80 ml. of chloroform. Separate the aqueous phase, which will have been diluted by transfers and other washings, and heat at 100° for 5 minutes. Cool and add 5 ml. of 0.5% solution of sodium metaperiodate and let stand for 20 minutes. Add 3 ml. of 3% zinc acetate solution to precipitate excess periodate and centrifuge. Decant the supernatant layer into 25 ml. of chloroform. Shake and allow to separate.

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[65] P. Formijne, L. Helt, and C. F. G. Lehr, *Koninkl. Ned. Akad. Wetenschap., Proc. Ser. C,* **59,** 31-7 (1956).

[66] P. Formijne and C. F. G. Lehr, *Clin. Chim. Acta* **4,** 302-6 (1959).

[67] E. W. McChesney, J. M. Shekosky, W. Eckert, and R. F. Koss, *J. Am. Pharm. Assoc., Sci. Ed.* **49,** 28-32 (1960).

Evaporate a 20-ml. aliquot of the chloroform layer to dryness and add 3 ml. of a fresh solution containing 1 mg. of *p*-nitrophenylhydrazine in 5% perchloric acid solution. Heat at 100° for 10 minutes and cool. Add exactly 8 ml. of 1:2 methylene chloride-*n*-hexane. Shake for 1-2 minutes and draw off 5 ml. of the solvent phase. Add 2 ml. of a 1:3 mixture of 10% tetraethylammonium hydroxide and absolute methanol. Mix well to convert the *p*-nitrophenylhydrazone to the quinoid salt form. Read the methanol phase at 570 mμ.

Unchanged dextrosulfendiol. Mix a 5-ml. sample with 15 ml. of absolute methanol. Extract with 80 ml. of *n*-hexane and discard the extract. Extract the methanol phase to dryness and take up with 25 ml. of 0.4 *M* phosphate buffer of pH 6.2. Extract with 25 ml. of ethyl acetate and discard the aqueous phase. Wash the solvent phase with 10 ml. of the buffer. Evaporate the ethyl acetate solution to dryness and take up in 18 ml. of water. Add 2 ml. of 4% sodium hydroxide solution. Digest at 100° for 30 minutes and cool. Proceed as for total dextrosulfendiol from "Add 3 ml. of 20% solution of. . . ."

Serum. Unchanged dextrosulfendiol. Mix 3 ml. of serum with 7 ml. of 20% solution of sodium dihydrogen phosphate. Shake for 15 minutes with 75 ml. of ethyl acetate. Centrifuge and evaporate 70 ml. of the solvent layer. Take up the residue in 30 ml. of 75% methanol and extract with 60 ml. of *n*-hexane. Discard the hexane and evaporate the methanol layer. Proceed as for unchanged dextrosulfendiol in urine from "Add 2 ml. of 4% sodium hydroxide solution. . . ."

Tissue. Homogenize 25 grams of tissue with 85 ml. of water and 15 ml. of 20% sodium dihydrogen phosphate solution. Extract for 15 minutes with 500 ml. of ethyl acetate. Wash the solvent layer with 40 ml. of 0.4 *M* buffer for pH 6.2. Add 5 grams of Filter-cel to the solvent layer and filter. Evaporate 425 ml. of the solvent layer to dryness. Take up in 75 ml. of 75% methanol and extract with 150 ml. and 150 ml. of *n*-hexane. If not now clear extract again. Evaporate the methanol layer substantially to dryness and dilute to 18 ml. Add 2 ml. of 4% sodium hydroxide solution. Digest at 100° for 30 minutes and cool. Complete as for total dextrosulfendiol in urine from "Add 3 ml. of 20% solution of. . . ."

Feces. Mix 3 grams of dried feces, 20 ml. of water, 5 ml. of 20% sodium dihydrogen phosphate solution, and 125 ml. of ethyl acetate.

Shake for 20 minutes and let separate. Evaporate 100 ml. of the solvent layer to dryness. Take up the residue in 75 ml. of 75% methanol and extract with 150 ml. and 150 ml. of hexane. Discard the hexane extracts and evaporate the methanol solution to dryness. Proceed as for total dextrosulfendiol from "Add 3 ml. of 20% solution of. . . ."

Eggs. Homogenize the edible part of the egg with 400 ml. of acetone. Digest the homogenate at 100° for 15 minutes and cool. Filter and wash the residue with acetone. Evaporate the acetone solution to dryness and take up in 75 ml. of 75% methanol. Extract with 300 ml. and 300 ml. of hexane and discard the washings. Evaporate the methanol solution to dryness. Complete as for total dextrosulfendiol from "Add 3 ml. of 20% solution of. . . ."

Raceophenidol. Poultry feed. Shake 5 grams of feed with 100 ml. of acetone for 10 minutes and filter. Evaporate 75 ml. of clear filtrate to dryness. Add 25 ml. of 0.4 M phosphate buffer for pH 6.2 to the residue. Add exactly 125 ml. of ethyl acetate. Shake for 5 minutes and discard the aqueous phase. Add 5 grams of filter cel to the solvent phase and filter. Evaporate 100 ml. of the filtrate to dryness and proceed as for total dextrosulfendiol from "Add 3 ml. of 20% solution of. . . ." Read at 570 mμ and 700 mμ. Evaluate from the average of these two readings.

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